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(71) Applicant: THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC. [US/US]; Suite 300, 505 Coast Boulevard South, La Jolla, CA 92037-4641 (US).

(72) Inventors: HARPOLD, Michael, M.; 15630 Creek Hills, El Cajon, CA 92021 (US). ELLIS, Steven, B.; 8939 Oviedo Street, San Diego, CA 92129 (US). WILLIAMS, Mark, E.; 6919 Pear Tree Drive, Carlsbad, CA 92009 (US). McCUE, Ann, F.; 6939 Colorado Avenue, La Mesa, CA 91942 (US). GILLESPIE, Alison; 12922 Texana Street, San Diego, CA 92129 (US).

(74) Agents: SEIDMAN, Stephanie et al.; Brown, Martin, Haller & McClain, 1660 Union Street, San Diego, CA 92101-2926 (US).

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(54) Title: HUMAN CALCIUM CHANNEL COMPOSITIONS AND METHODS USING THEM

(57) Abstract

Isolated DNA encoding each of human calcium chanel α_1-, α_2-, β- and γ-subunits, including subunits that arise as splice variants of primary transcripts, is provided. In particular DNA clones encoding each of the $\alpha_{1A\text{-}1}$, $\alpha_{1A\text{-}2}$, $\alpha_{1E\text{-}1}$, $\alpha_{1C\text{-}2}$, $\alpha_{1E\text{-}3}$, $\beta_{3\text{-}1}$, β_{2C}, β_{2D}, β_{2E} and β_4 subunits of human calcium channels are provided. Cells and vectors containing the DNA, subunit specific antibodies and nucleic acid probes and methods for identifying compounds that modulate the activity of human calcium channels are also provided.

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HUMAN CALCIUM CHANNEL COMPOSITIONS AND METHODS USING THEM

TECHNICAL FIELD

The present invention relates to molecular biology and pharmacology. More particularly, the invention relates to calcium channel compositions and methods of making and using the same.

BACKGROUND OF THE INVENTION

Calcium channels are membrane-spanning, multi-subunit proteins that allow controlled entry of Ca^{2+} ions into cells from the extracellular fluid. Cells throughout the animal kingdom, and at least some bacterial, fungal and plant cells, possess one or more types of calcium channel.

The most common type of calcium channel is voltage dependent. "Opening" of a voltage-dependent channel to allow an influx of Ca^{2+} ions into the cells requires a depolarization to a certain level of the potential difference between the inside of the cell bearing the channel and the extracellular medium bathing the cell. The rate of influx of Ca^{2+} into the cell depends on this potential difference. All "excitable" cells in animals, such as neurons of the central nervous system (CNS), peripheral nerve cells and muscle cells, including those of skeletal muscles, cardiac muscles, and venous and arterial smooth muscles, have voltage-dependent calcium channels.

Multiple types of calcium channels have been identified in mammalian cells from various tissues, including skeletal muscle, cardiac muscle, lung, smooth muscle and brain, [see, *e.g.*, Bean, B.P.(1989) *Ann. Rev. Physiol.* 51:367-384 and Hess, P. (1990) *Ann. Rev. Neurosci.* 56:337]. The different types of calcium channels have been broadly categorized into four classes, L-, T-, N-, and P-type, distinguished by current kinetics, holding potential sensitivity and sensitivity to calcium channel agonists and antagonists.

Calcium channels are multisubunit proteins that contain two large subunits, designated α_1 and α_2, which have molecular weights between about 130 and about 200 kilodaltons ("kD"),

and one to three different smaller subunits of less than about 60 kD in molecular weight. At least one of the larger subunits and possibly some of the smaller subunits are glycosylated. Some of the subunits are capable of being phosphorylated. The α_1 subunit has a molecular weight of about 150 to about 170 kD when analyzed by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) after isolation from mammalian muscle tissue and has specific binding sites for various 1,4-dihydropyridines (DHPs) and phenylalkylamines. Under non-reducing conditions (in the presence of N-ethylmaleimide), the α_2 subunit migrates in SDS-PAGE as a band corresponding to a molecular weight of about 160-190 kD. Upon reduction, a large fragment and smaller fragments are released. The β subunit of the rabbit skeletal muscle calcium channel is a phosphorylated protein that has a molecular weight of 52-65 kD as determined by SDS-PAGE analysis. This subunit is insensitive to reducing conditions. The γ subunit of the calcium channel, which is not observed in all purified preparations, appears to be a glycoprotein with an apparent molecular weight of 30-33 kD, as determined by SDS-PAGE analysis.

In order to study calcium channel structure and function, large amounts of pure channel protein are needed. Because of the complex nature of these multisubunit proteins, the varying concentrations of calcium channels in tissue sources of the protein, the presence of mixed populations of calcium channels in tissues, difficulties in obtaining tissues of interest, and the modifications of the native protein that can occur during the isolation procedure, it is extremely difficult to obtain large amounts of highly purified, completely intact calcium channel protein.

Characterization of a particular type of calcium channel by analysis of whole cells is severely restricted by the presence of mixed populations of different types of calcium channels in the majority of cells. Single-channel recording methods that are used to examine individual calcium channels

do not reveal any information regarding the molecular structure or biochemical composition of the channel. Furthermore, in performing this type of analysis, the channel is isolated from other cellular constituents that might be important for natural functions and pharmacological interactions.

Characterization of the gene or genes encoding calcium channels provides another means of characterization of different types of calcium channels. The amino acid sequence determined from a complete nucleotide sequence of the coding region of a gene encoding a calcium channel protein represents the primary structure of the protein. Furthermore, secondary structure of the calcium channel protein and the relationship of the protein to the membrane may be predicted based on analysis of the primary structure. For instance, hydropathy plots of the α_1 subunit protein of the rabbit skeletal muscle calcium channel indicate that it contains four internal repeats, each containing six putative transmembrane regions [Tanabe, T. *et al.* (1987) *Nature 328*:313].

Because calcium channels are present in various tissues and have a central role in regulating intracellular calcium ion concentrations, they are implicated in a number of vital processes in animals, including neurotransmitter release, muscle contraction, pacemaker activity, and secretion of hormones and other substances. These processes appear to be involved in numerous human disorders, such as CNS and cardiovascular diseases. Calcium channels, thus, are also implicated in numerous disorders. A number of compounds useful for treating various cardiovascular diseases in animals, including humans, are thought to exert their beneficial effects by modulating functions of voltage-dependent calcium channels present in cardiac and/or vascular smooth muscle. Many of these compounds bind to calcium channels and block, or reduce the rate of, influx of Ca^{2+} into the cells in response to depolarization of the cell membrane.

The results of studies of recombinant expression of rabbit calcium channel α_1 subunit-encoding cDNA clones and transcripts of the cDNA clones indicate that the α_1 subunit forms the pore through which calcium enters cells. The relevance of the barium currents generated in these recombinant cells to the actual current generated by calcium channels containing as one component the respective α_1 subunits *in vivo* is unclear. In order to completely and accurately characterize and evaluate different calcium channel types, however, it is essential to examine the functional properties of recombinant channels containing all of the subunits as found *in vivo*.

In order to conduct this examination and to fully understand calcium channel structure and function, it is critical to identify and characterize as many calcium channel subunits as possible. Also in order to prepare recombinant cells for use in identifying compounds that interact with calcium channels, it is necessary to be able to produce cells that express uniform populations of calcium channels containing defined subunits.

An understanding of the pharmacology of compounds that interact with calcium channels in other organ systems, such as the CNS, may aid in the rational design of compounds that specifically interact with subtypes of human calcium channels to have desired therapeutic effects, such as in the treatment of neurodegenerative and cardiovascular disorders. Such understanding and the ability to rationally design therapeutically effective compounds, however, have been hampered by an inability to independently determine the types of human calcium channels and the molecular nature of individual subtypes, particularly in the CNS, and by the unavailability of pure preparations of specific channel subtypes to use for evaluation of the specificity of calcium channel-effecting compounds. Thus, identification of DNA encoding human calcium channel subunits and the use of such DNA for expression of calcium channel subunits and functional

calcium channels would aid in screening and designing therapeutically effective compounds.

Therefore, it is an object herein, to provide DNA encoding specific calcium channel subunits and to provide eukaryotic cells bearing recombinant tissue-specific or subtype- specific calcium channels. It is also an object to provide assays for identification of potentially therapeutic compounds that act as calcium channel antagonists and agonists.

SUMMARY OF THE INVENTION

Isolated and purified nucleic acid fragments that encode human calcium channel subunits are provided. DNA encoding α_1 subunits of a human calcium channel, and RNA, encoding such subunits, made upon transcription of such DNA are provided. In particular, DNA fragments encoding α_1 subunits of voltage-dependent human calcium channels (VDCCs) type A, type B (also referred to as VDCC IV), type C (also referred to as VDCC II) type D (also referred to as VDCC III) and type E are provided.

DNA encoding α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} subunits is provided. DNA encoding an α_{1D} subunit that includes the amino acids substantially as set forth as residues 10-2161 of SEQ ID No. 1 is provided. DNA encoding an α_{1D} subunit that includes substantially the amino acids set forth as amino acids 1-34 in SEQ ID No. 2 in place of amino acids 373-406 of SEQ ID No. 1 is also provided. DNA encoding an α_{1C} subunit that includes the amino acids substantially as set forth in SEQ ID No. 3 or SEQ ID No. 6 and DNA encoding an α_{1B} subunit that includes an amino acid sequence substantially as set forth in SEQ ID No. 7 or in SEQ ID No. 8 is also provided.

DNA encoding α_{1A} subunits is also provided. Such DNA includes DNA encoding an α_{1A} subunit that has substantially the same sequence of amino acids as encoded by the DNA set forth in SEQ ID No. 22 or No. 23 or other splice variants of α_{1A} that include all or part of the sequence set forth in SEQ ID No. 22 or 23. The sequence set forth in SEQ ID NO. 22 is a splice variant designated α_{1A-1}; and the sequence set forth in SEQ ID NO. 23 is a splice variant designated α_{1A-2}. DNA encoding α_{1A} subunits also include DNA encoding subunits that can be isolated using all or a portion of the DNA having SEQ ID NO. 21, 22 or 23 or DNA obtained from the phage lysate of an *E. coli* host containing DNA encoding an α_{1A} subunit that has been deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under Accession No. 75293 in accord with the Budapest Treaty. The

DNA in such phage includes a DNA fragment having the sequence set forth in SEQ ID No. 21. This fragment selectively hybridizes under conditions of high stringency to DNA encoding α_{1A} but not to DNA encoding α_{1B} and, thus, can be used to isolate DNA that encodes α_{1A} subunits.

DNA encoding α_{1E} subunits of a human calcium channel is also provided. This DNA includes DNA that encodes an α_{1E} splice variant designated α_{1E-1} encoded by the DNA set forth in SEQ ID No. 24, and a variant designated α_{1E-3} encoded by SEQ ID No. 25. This DNA also includes other splice variants thereof that encodes sequences of amino acids encoded by all or a portion of the sequences of nucleotides set forth in SEQ ID Nos. 24 and 25 and DNA that hybridizes under conditions of high stringency to the DNA of SEQ ID. No. 24 or 25 and that encodes an α_{1E} splice variant.

DNA encoding α_2 subunits of a human calcium channel, and RNA encoding such subunits, made upon transcription of such a DNA are provided. DNA encoding splice variants of the α_2 subunit, including tissue-specific splice variants, are also provided. In particular, DNA encoding the α_{2a}-α_{2e} subunit subtypes is provided. In particularly preferred embodiments, the DNA encoding the α_2 subunit that is produced by alternative processing of a primary transcript that includes DNA encoding the amino acids set forth in SEQ ID 11 and the DNA of SEQ ID No. 13 inserted between nucleotides 1624 and 1625 of SEQ ID No. 11 is provided. The DNA and amino acid sequences of α_{2a} - α_{2e} are set forth in SEQ. ID Nos. 11 and 29-32, respectively.

Isolated and purified DNA fragments encoding human calcium channel β subunits, including DNA encoding β_1, β_2, β_3 and β_4 subunits, and splice variants of the β subunits are provided. RNA encoding β subunits, made upon transcription of the DNA is also provided.

DNA encoding a β_1 subunit that is produced by alternative processing of a primary transcript that includes DNA encoding the amino acids set forth in SEQ ID No. 9, but including the

DNA set forth in SEQ ID No. 12 inserted in place of nucleotides 615-781 of SEQ ID No. 9 is also provided. DNA encoding β_1 subunits that are encoded by transcripts that have the sequence set forth in SEQ ID No. 9 including the DNA set forth in SEQ ID No. 12 inserted in place of nucleotides 615-781 of SEQ ID No. 9, but that lack one or more of the following sequences of nucleotides: nucleotides 14-34 of SEQ ID No. 12, nucleotides 13-34 of SEQ ID No. 12, nucleotides 35-55 of SEQ ID No 12, nucleotides 56-190 of SEQ ID No. 12 and nucleotides 191-271 of SEQ ID No. 12 are also provided. In particular, β_1 subunit splice variants β_{1-1}-β_{1-5} (see, SEQ ID Nos. 9, 10 and 33-35) described below, are provided.

B_2 subunit splice variants β_{2c}-β_{2e}, that include all or a portion of SEQ ID Nos. 26, 29 and 30 are provided; β_3 subunit splice variants, including β_3 subunit splice variants that have the sequences set forth in SEQ ID Nos 19 and 20, and DNA encoding the β_4 subunit that includes DNA having the sequence set forth in SEQ ID No. 27 and the amino acid sequence set forth in SEQ ID No. 28 are provided.

Also *Escherichia coli* (*E. coli*) host cells harboring plasmids containing DNA encoding β_3 have been deposited in accord with the Budapest Treaty under Accession No. 69048 at the American Type Culture Collection. The deposited clone encompasses nucleotides 122-457 in SEQ ID No. 19 and 107-443 in SEQ ID No. 20.

DNA encoding β subunits that are produced by alternative processing of a primary transcript encoding a β subunit, including a transcript that includes DNA encoding the amino acids set forth in SEQ ID No. 9 or including a primary transcript that encodes β_3 as deposited under ATCC Accession No. 69048, but lacking and including alternative exons are provided or may be constructed from the DNA provided herein.

DNA encoding γ subunits of human calcium channels is also provided. RNA, encoding γ subunits, made upon transcription of the DNA are also provided. In particular, DNA containing

the sequence of nucleotides set forth in SEQ ID No. 14 is provided.

Full-length DNA clones and corresponding RNA transcripts, encoding α_1, including splice variants of α_{1A}, α_{1D}, α_{1B}, α_{1C}, and α_{1E}, α_2 and β subunits, including β_{1-1}-β_{1-5}, β_{2C}, β_{2D}, β_{2E}, β_{3-1} and β_4 of human calcium channels are provided. Also provided are DNA clones encoding a substantial portions of the certain α_{1C} subtype subunits and γ subunits of voltage-dependent human calcium channels for the preparation of full-length DNA clones encoding the corresponding full-length subunits. Full-length clones may be readily obtained using the disclosed DNA as a probe as described herein.

The the α_{1A} subunit, α_{1C} subunit, α_{1E} subunit and splice variants thereof, the β_{2D}, β_{2C} and β_{2E} subunits and β_4 subunits and nucleic acids encoding these subunits are of particular interest herein.

Eukaryotic cells containing heterologous DNA encoding one or more calcium channel subunits, particularly human calcium channel subunits, or containing RNA transcripts of DNA clones encoding one or more of the subunits are provided. A single α_1 subunit can form a channel. The requisite combination of subunits for formation of active channels in selected cells, however, can be determined empirically using the methods herein. For example, if a selected α_1 subtype or variant does not form an active channel in a selected cell line, an additional subunit or subunits can be added until an active channel is formed.

In preferred embodiments, the cells contain DNA or RNA encoding a human α_1 subunit, preferably at least an α_{1D}, α_{1B}, α_{1A} or α_{1E} subunit. In more preferred embodiments, the cells contain DNA or RNA encoding additional heterologous subunits, including at least one β, α_2 or γ subunit. In such embodiments, eukaryotic cells stably or transiently transfected with any combination of one, two, three or four of the subunit-encoding DNA clones, such as DNA encoding any of α_1, $\alpha_1 + \beta$, $\alpha_1 + \beta + \alpha_2$, are provided.

The eukaryotic cells provided herein contain heterologous DNA that encodes an α_1 subunit or heterologous DNA that encodes an α_1 subunit and heterologous DNA that encodes a β subunit. At least one subunit is selected α_{1A-1}, α_{1A-2}, α_{1C-2}, α_{1E-1}, α_{1E-3}, β_{2C}, β_{2D}, β_{2F}, a β_{3-1}, β_{3-2} subunit or a β_4 subunit. In preferred embodiments, the cells express such heterologous calcium channel subunits and include one or more of the subunits in membrane-spanning heterologous calcium channels. In more preferred embodiments, the eukaryotic cells express functional, heterologous calcium channels that are capable of gating the passage of calcium channel-selective ions and/or binding compounds that, at physiological concentrations, modulate the activity of the heterologous calcium channel. In certain embodiments, the heterologous calcium channels include at least one heterologous calcium channel subunit. In most preferred embodiments, the calcium channels that are expressed on the surface of the eukaryotic cells are composed substantially or entirely of subunits encoded by the heterologous DNA or RNA. In preferred embodiments, the heterologous calcium channels of such cells are distinguishable from any endogenous calcium channels of the host cell. Such cells provide a means to obtain homogeneous populations of calcium channels. Typically, the cells contain the selected calcium channel as the only heterologous ion channel expressed by the cell.

In certain embodiments the recombinant eukaryotic cells that contain the heterologous DNA encoding the calcium channel subunits are produced by transfection with DNA encoding one or more of the subunits or are injected with RNA transcripts of DNA encoding one or more of the calcium channel subunits. The DNA may be introduced as a linear DNA fragment or may be included in an expression vector for stable or transient expression of the subunit-encoding DNA. Vectors containing DNA encoding human calcium channel subunits are also provided.

The eukaryotic cells that express heterologous calcium channels may be used in assays for calcium channel function or, in the case of cells transformed with fewer subunit-encoding nucleic acids than necessary to constitute a functional recombinant human calcium channel, such cells may be used to assess the effects of additional subunits on calcium channel activity. The additional subunits can be provided by subsequently transfecting such a cell with one or more DNA clones or RNA transcripts encoding human calcium channel subunits.

The recombinant eukaryotic cells that express membrane spanning heterologous calcium channels may be used in methods for identifying compounds that modulate calcium channel activity. In particular, the cells are used in assays that identify agonists and antagonists of calcium channel activity in humans and/or assessing the contribution of the various calcium channel subunits to the transport and regulation of transport of calcium ions. Because the cells constitute homogeneous populations of calcium channels, they provide a means to identify agonists or antagonists of calcium channel activity that are specific for each such population.

The assays that use the eukaryotic cells for identifying compounds that modulate calcium channel activity are also provided. In practicing these assays the eukaryotic cell that expresses a heterologous calcium channel, containing at least on subunit encoded by the DNA provided herein, is in a solution containing a test compound and a calcium channel selective ion, the cell membrane is depolarized, and current flowing into the cell is detected. If the test compound is one that modulates calcium channel activity, the current that is detected is different from that produced by depolarizing the same or a substantially identical cell in the presence of the same calcium channel-selective ion but in the absence of the compound. In preferred embodiments, prior to the depolarization step, the cell is maintained at a holding potential which substantially inactivates calcium channels

which are endogenous to the cell. Also in preferred embodiments, the cells are mammalian cells, most preferably HEK cells, or amphibian oöcytes.

Nucleic acid probes, typically labeled for detection, containing at least about 14, preferably 16, or, if desired, 20 or 30 or more, contiguous nucleotides of α_{1D}, α_{1C}, α_{1B}, α_{1A} and α_{1E}, α_2, β, including β_1, β_2 ,β_3 and β_4 splice variants and γ subunit-encoding DNA are provided. Methods using the probes for the isolation and cloning of calcium channel subunit-encoding DNA, including splice variants within tissues and inter-tissue variants are also provided.

Purified human calcium channel subunits and purified human calcium channels are provided. The subunits and channels can be isolated from a eukaryotic cell transfected with DNA that encodes the subunit.

In another embodiment, immunoglobulins or antibodies obtained from the serum of an animal immunized with a substantially pure preparation of a human calcium channel, human calcium channel subunit or epitope-containing fragment of a human calcium subunit are provided. Monoclonal antibodies produced using a human calcium channel, human calcium channel subunit or epitope-containing fragment thereof as an immunogen are also provided. *E. coli* fusion proteins including a fragment of a human calcium channel subunit may also be used as immunogen. Such fusion proteins may contain a bacterial protein or portion thereof, such as the *E. coli* TrpE protein, fused to a calcium channel subunit peptide. The immunoglobulins that are produced using the calcium channel subunits or purified calcium channels as immunogens have, among other properties, the ability to specifically and preferentially bind to and/or cause the immunoprecipitation of a human calcium channel or a subunit thereof which may be present in a biological sample or a solution derived from such a biological sample. Such antibodies may also be used to selectively isolate cells that express calcium channels that contain the subunit for which the antibodies are specific.

Methods for modulating the activity of ion channels by contacting the calcium channels with an effective amount of the above-described antibodies are also provided.

A diagnostic method for determining the presence of Lambert Eaton Syndrome (LES) in a human based on immunological reactivity of LES immunoglobulin G (IgG) with a human calcium channel subunit or a eukaryotic cell which expresses a recombinant human calcium channel or a subunit thereof is also provided. In particular, an immunoassay method for diagnosing Lambert-Eaton Syndrome in a person by combining serum or an IgG fraction from the person (test serum) with calcium channel proteins, including the α and β subunits, and ascertaining whether antibodies in the test serum react with one or more of the subunits, or a recombinant cell which expresses one or more of the subunits to a greater extent than antibodies in control serum, obtained from a person or group of persons known to be free of the Syndrome, is provided. Any immunoassay procedure known in the art for detecting antibodies against a given antigen in serum can be employed in the method.

DETAILED DESCRIPTION OF THE INVENTION

Definitions:

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents and publications referred to herein are incorporated by reference herein.

Reference to each of the calcium channel subunits includes the subunits that are specifically disclosed herein and human calcium channel subunits encoded by DNA that can be isolated by using the DNA disclosed as probes and screening an appropriate human cDNA or genomic library under at least low stringency. Such DNA also includes DNA that encodes proteins that have about 40% homology to any of the subunits proteins described herein or DNA that hybridizes under conditions of at least low stringency to the DNA provided herein and the

protein encoded by such DNA exhibits additional identifying characteristics, such as function or molecular weight.

It is understood that subunits that are encoded by transcripts that represent splice variants of the disclosed subunits or other such subunits may exhibit less than 40% overall homology to any single subunit, but will include regions of such homology to one or more such subunits. It is also understood that 40% homology refers to proteins that share approximately 40% of their amino acids in common or that share somewhat less, but include conservative amino acid substitutions, whereby the activity of the protein is not substantially altered.

As used herein, the α_1 subunits types, encoded by different genes, are designated as type α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E}. These types have also been referred to as VDCC IV for α_{1B}, VDCC II for α_{1C} and VDCC III for α_{1D}. Subunit subtypes, which are splice variants, are referred to, for example as α_{1B-1}, α_{1B-2}, α_{1C-1} etc.

Thus, as used herein, DNA encoding the α_1 subunit refers to DNA that hybridizes to the DNA provided herein under conditions of at least low stringency or encodes a subunit that has at least about 40% homology to protein encoded by DNA disclosed herein that encodes an α_1 subunit of a human calcium. An α_1 subunit may be identified by its ability to form a calcium channel. Typically, α_1 subunits have molecular masses greater than at least about 120 kD. Also, hydropathy plots of deduced α_1 subunit amino acid sequences indicate that the α_1 subunits contain four internal repeats, each containing six putative transmembrane domains.

The activity of a calcium channel may be assessed *in vitro* by methods known to those of skill in the art, including the electrophysiological and other methods described herein. Typically, α_1 subunits include regions to which one or more modulators of calcium channel activity, such as a 1,4-DHP or ω-CgTx, interact directly or indirectly. Types of α_1 subunits may be distinguished by any method known to those of skill in

the art, including on the basis of binding specificity. For example, it has been found herein that α_{1B} subunits participate in the formation channels that have previously been referred to as N-type channels, α_{1D} subunits participate in the formation of channels that had previously been referred to as L-type channels, and α_{1A} subunits appear to participate in the formation of channels that exhibit characteristics typical of channels that had previously been designated P-type channels. Thus, for example, the activity of channels that contain the α_{1B} subunit are insensitive to 1,4-DHPs; whereas the activity of channels that contain the α_{1D} subunit are modulated or altered by a 1,4-DHP. It is presently preferable to refer to calcium channels based on pharmacological characteristics and current kinetics and to avoid historical designations. Types and subtypes of α_1 subunits may be characterized on the basis of the effects of such modulators on the subunit or a channel containing the subunit as well as differences in currents and current kinetics produced by calcium channels containing the subunit.

As used herein, an α_2 subunit is encoded by DNA that hybridizes to the DNA provided herein under conditions of low stringency or encodes a protein that has at least about 40% homology with that disclosed herein. Such DNA encodes a protein that typically has a molecular mass greater than about 120 kD, but does not form a calcium channel in the absence of an α_1 subunit, and may alter the activity of a calcium channel that contains an α_1 subunit. Subtypes of the α_2 subunit that arise as splice variants are designated by lower case letter, such as α_{2a}, . . . α_{2e}. In addition, the α_2 subunit and the large fragment produced when the protein is subjected to reducing conditions appear to be glycosylated with at least N-linked sugars and do not specifically bind to the 1,4-DHPs and phenylalkylamines that specifically bind to the α_1 subunit. The smaller fragment, the C-terminal fragment, is referr d to as the δ subunit and includes amino acids from about 946 (SEQ ID No. 11) through about the C-terminus. This

fragment may dissociate from the remaining portion of α_2 when the α_2 subunit is exposed to reducing conditions.

As used herein, a β subunit is encoded by DNA that hybridizes to the DNA provided herein under conditions of low stringency or encodes a protein that has at least about 40% homology with that disclosed herein and is a protein that typically has a molecular mass lower than the α subunits and on the order of about 50-80 kD, does not form a detectable calcium channel in the absence of an α_1 subunit, but may alter the activity of a calcium channel that contains an α_1 subunit or that contains an α_1 and α_2 subunit.

Types of the β subunit that are encoded by different genes are designated with subscripts, such as β_1, β_2, β_3 and β_4. Subtypes of β subunits that arise as splice variants of a particular type are designated with a numerical subscript referring to the type and to the variant. Such subtypes include, but are not limited to the β_1 splice variants, including β_{1-1}-β_{1-5} and β_2 variants, including β_{2C}-β_{2E}.

As used herein, a γ subunit is a subunit encoded by DNA disclosed herein as encoding the γ subunit and may be isolated and identified using the DNA disclosed herein as a probe by hybridization or other such method known to those of skill in the art, whereby full-length clones encoding a γ subunit may be isolated or constructed. A γ subunit will be encoded by DNA that hybridizes to the DNA provided herein under conditions of low stringency or exhibits sufficient sequence homology to encode a protein that has at least about 40% homology with the γ subunit described herein.

Thus, one of skill in the art, in light of the disclosure herein, can identify DNA encoding α_1, α_2, β, δ and γ calcium channel subunits, including types encoded by different genes and subtypes that represent splice variants. For example, DNA probes based on the DNA disclosed herein may be used to screen an appropriate library, including a genomic or cDNA library, for hybridization to the probe and obtain DNA in one or more clones that includes an open reading fragment that

encodes an entire protein. Subsequent to screening an appropriate library with the DNA disclosed herein, the isolated DNA can be examined for the presence of an open reading frame from which the sequence of the encoded protein may be deduced. Determination of the molecular weight and comparison with the sequences herein should reveal the identity of the subunit as an α_1, α_2 etc. subunit. Functional assays may, if necessary, be used to determine whether the subunit is an α_1, α_2 subunit or β subunit.

For example, DNA encoding an α_{1A} subunit may be isolated by screening an appropriate library with DNA, encoding all or a portion of the human α_{1A} subunit. Such DNA includes the DNA in the phage deposited under ATCC Accession No. 75293 that encodes a portion of an α_1 subunit. DNA encoding an α_{1A} subunit may obtained from an appropriate library by screening with an oligonucleotide having all or a portion of the sequence set forth in SEQ ID No. 21, 22 and/or 23 or with the DNA in the deposited phage. Alternatively, such DNA may have a sequence that encodes an α_{1A} subunit that is encoded by SEQ ID NO. 22 or 23.

Similarly, DNA encoding β_3 may be isolated by screening a human cDNA library with DNA probes prepared from the plasmid β1.42 deposited under ATCC Accession No. 69048 or obtained from an appropriate library using probes having sequences prepared according to the sequences set forth in SEQ ID Nos. 19 and/or 20. Also, DNA encoding β_4 may be isolated by screening a human cDNA library with DNA probes prepared according to DNA set forth in SEQ ID No. 27, which sets forth the DNA sequence of a clone encoding a β_4 subunit. The amino acid sequence is set forth in SEQ ID No. 28. Any method known to those of skill in the art for isolation and identification of DNA and preparation of full-length genomic or cDNA clones, including methods exemplified herein, may be used. DNA encoding

The subunit encoded by isolated DNA may be identified by comparison with the DNA and amino acid sequences of the

subunits provided herein. Splice variants share extensive regions of homology, but include non-homologous regions, subunits encoded by different genes share a uniform distribution of non-homologous sequences.

As used herein, a splice variant refers to a variant produced by differential processing of a primary transcript of genomic DNA that results in more than one type of mRNA. Splice variants may occur within a single tissue type or among tissues (tissue-specific variants). Thus, cDNA clones that encode calcium channel subunit subtypes that have regions of identical amino acids and regions of different amino acid sequences are referred to herein as "splice variants".

As used herein, a "calcium channel-selective ion" is an ion that is capable of flowing through, or being blocked from flowing through, a calcium channel which spans a cellular membrane under conditions which would substantially similarly permit or block the flow of Ca^{2+}. Ba^{2+} is an example of an ion which is a calcium channel-selective ion.

As used herein, a compound that modulates calcium channel activity is one that affects the ability of the calcium channel to pass calcium channel-selective ions or affects other detectable calcium channel features, such as current kinetics. Such compounds include calcium channel antagonists and agonists and compounds that exert their effect on the activity of the calcium channel directly or indirectly.

As used herein, a "substantially pure" subunit or protein is a subunit or protein that is sufficiently free of other polypeptide contaminants to appear homogeneous by SDS-PAGE or to be unambiguously sequenced.

As used herein, selectively hybridize means that a DNA fragment hybridizes to a second fragment with sufficient specificity to permit the second fragment to be identified or isolated from among a plurality of fragments. In general, selective hybridization occurs at conditions of high stringency.

As used herein, heterologous or foreign DNA and RNA are used interchangeably and refer to DNA or RNA that does not occur naturally as part of the genome in which it is present or which is found in a location or locations in the genome that differ from that in which it occurs in nature. It is DNA or RNA that is not endogenous to the cell and has been artificially introduced into the cell. Examples of heterologous DNA include, but are not limited to, DNA that encodes a calcium channel subunit and DNA that encodes RNA or proteins that mediate or alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. The cell that expresses the heterologous DNA, such as DNA encoding a calcium channel subunit, may contain DNA encoding the same or different calcium channel subunits. The heterologous DNA need not be expressed and may be introduced in a manner such that it is integrated into the host cell genome or is maintained episomally.

As used herein, operative linkage of heterologous DNA to regulatory and effector sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences, refers to the functional relationship between such DNA and such sequences of nucleotides. For example, operative linkage of heterologous DNA to a promoter refers to the physical and functional relationship between the DNA and the promoter such that the transcription of such DNA is initiated from the promoter by an RNA polymerase that specifically recognizes, binds to and transcribes the DNA in reading frame.

As used herein, isolated, substantially pure DNA refers to DNA fragments purified according to standard techniques employed by those skilled in the art [see, *e.g.*, Maniatis *et al.* (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY].

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into

peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression may, if an appropriate eukaryotic host cell or organism is selected, include splicing of the mRNA.

As used herein, vector or plasmid refers to discrete elements that are used to introduce heterologous DNA into cells for either expression of the heterologous DNA or for replication of the cloned heterologous DNA. Selection and use of such vectors and plasmids are well within the level of skill of the art.

As used herein, expression vector includes vectors capable of expressing DNA fragments that are in operative linkage with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or may integrate into the host cell genome.

As used herein, a promoter region refers to the portion of DNA of a gene that controls transcription of DNA to which it is operatively linked. The promoter region includes specific sequences of DNA that are sufficient for RNA polymerase recognition, binding and transcription initiation. This portion of the promoter region is referred to as the promoter. In addition, the promoter region includes sequences that modulate this recognition, binding and transcription initiation activity of the RNA polymerase. These sequences may be *cis* acting or may be responsive to *trans* acting factors. Promoters, depending upon the nature of the regulation, may be constitutive or regulated.

As used herein, a recombinant eukaryotic cell is a eukaryotic cell that contains heterologous DNA or RNA.

As used herein, a recombinant or heterologous calcium channel refers to a calcium channel that contains one or more subunits that are encoded by heterologous DNA that has been introduced into and expressed in a eukaryotic cells that expresses the recombinant calcium channel. A recombinant calcium channel may also include subunits that are produced by DNA endogenous to the cell. In certain embodiments, the recombinant or heterologous calcium channel may contain only subunits that are encoded by heterologous DNA.

As used herein, "functional" with respect to a recombinant or heterologous calcium channel means that the channel is able to provide for and regulate entry of calcium channel-selective ions, including, but not limited to, Ca^{2+} or Ba^{2+}, in response to a stimulus and/or bind ligands with affinity for the channel. Preferably such calcium channel activity is distinguishable, such as electrophysiological, pharmacological and other means known to those of skill in the art, from any endogenous calcium channel activity that in the host cell.

As used herein, a peptide having an amino acid sequence substantially as set forth in a particular SEQ ID No. includes peptides that have the same function but may include minor variations in sequence, such as conservative amino acid changes or minor deletions or insertions that do not alter the activity of the peptide. The activity of a calcium channel receptor subunit peptide refers to its ability to form functional calcium channels with other such subunits.

As used herein, a physiological concentration of a compound is that which is necessary and sufficient for a biological process to occur. For example, a physiological concentration of a calcium channel-selective ion is a concentration of the calcium channel-selective ion necessary and sufficient to provide an inward current when the channels open.

As used herein, activity of a calcium channel refers to the movement of a calcium channel-selective ion through a

calcium channel. Such activity may be measured by any method known to those of skill in the art, including, but not limited to, measurement of the amount of current which flows through the recombinant channel in response to a stimulus.

As used herein, a "functional assay" refers to an assay that identifies functional calcium channels. A functional assay, thus, is an assay to assess function.

As understood by those skilled in the art, assay methods for identifying compounds, such as antagonists and agonists, that modulate calcium channel activity, generally requires comparison to a control. One type of a "control" cell or "control" culture is a cell or culture that is treated substantially the same as the cell or culture exposed to the test compound except that the control culture is not exposed to the test compound. Another type of a "control" cell or "control" culture may be a cell or a culture of cells which are identical to the transfected cells except the cells employed for the control culture do not express functional calcium channels. In this situation, the response of test cell to the test compound is compared to the response (or lack of response) of the calcium channel-negative cell to the test compound, when cells or cultures of each type of cell are exposed to substantially the same reaction conditions in the presence of the compound being assayed. For example, in methods that use patch clamp electrophysiological procedures, the same cell can be tested in the presence and absence of the test compound, by changing the external solution bathing the cell as known in the art.

It is also understood that each of the subunits disclosed herein may be modified by making conservative amino acid substitutions and the resulting modified subunits are contemplated herein. Suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-

essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224). Such substitutions are preferably, although not exclusively, made in accordance with those set forth in TABLE 1 as follows:

TABLE 1

Original residue	Conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions are also permissible and may be determined empirically or in accord with known conservative substitutions. Any such modification of the polypeptide may be effected by any means known to those of skill in this art. Mutation may be effected by any method known to those of skill in the art, including site-specific or site-directed mutagenesis of DNA encoding the protein and the use of DNA amplification methods using primers to introduce and amplify alterations in the DNA template.

Identification and isolation of DNA encoding human calcium channel subunits

Methods for identifying and isolating DNA encoding α_1, α_2, β and γ subunits of human calcium channels are provided.

Identification and isolation of such DNA may be accomplished by hybridizing, under appropriate conditions, at least low stringency whereby DNA that encodes the desired

subunit is isolated, restriction enzyme-digested human DNA with a labeled probe having at least 14, preferably 16 or more nucleotides and derived from any contiguous portion of DNA having a sequence of nucleotides set forth herein by sequence identification number. Once a hybridizing fragment is identified in the hybridization reaction, it can be cloned employing standard cloning techniques known to those of skill in the art. Full-length clones may be identified by the presence of a complete open reading frame and the identity of the encoded protein verified by sequence comparison with the subunits provided herein and by functional assays to assess calcium channel- forming ability or other function. This method can be used to identify genomic DNA encoding the subunit or cDNA encoding splice variants of human calcium channel subunits generated by alternative splicing of the primary transcript of genomic subunit DNA. For instance, DNA, cDNA or genomic DNA, encoding a calcium channel subunit may be identified by hybridization to a DNA probe and characterized by methods known to those of skill in the art, such as restriction mapping and DNA sequencing, and compared to the DNA provided herein in order to identify heterogeneity or divergence in the sequences of the DNA. Such sequence differences may indicate that the transcripts from which the cDNA was produced result from alternative splicing of a primary transcript, if the non-homologous and homologous regions are clustered, or from a different gene if the non-homologous regions are distributed throughout the cloned DNA.

Any suitable method for isolating genes using the DNA provided herein may be used. For example, oligonucleotides corresponding to regions of sequence differences have been used to isolate, by hybridization, DNA encoding the full-length splice variant and can be used to isolate genomic clones. A probe, based on a nucleotide sequence disclosed herein, which encodes at least a portion of a subunit of a human calcium channel, such as a tissue-specific exon, may be used as a probe to clone related DNA, to clone a full-length

cDNA clone or genomic clone encoding the human calcium channel subunit.

Labeled, including, but not limited to, radioactively or enzymatically labeled, RNA or single-stranded DNA of at least 14 substantially contiguous bases, preferably 16 or more, generally at least 30 contiguous bases of a nucleic acid which encodes at least a portion of a human calcium channel subunit, the sequence of which nucleic acid corresponds to a segment of a nucleic acid sequence disclosed herein by reference to a SEQ ID No. are provided. Such nucleic acid segments may be used as probes in the methods provided herein for cloning DNA encoding calcium channel subunits. See, generally, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press.

In addition, nucleic acid amplification techniques, which are well known in the art, can be used to locate splice variants of calcium channel subunits by employing oligonucleotides based on DNA sequences surrounding the divergent sequence primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice variants of transcripts encoding human calcium channel subunits.

DNA encoding types and subtypes of each of the α_1, α_2, β and γ subunit of voltage-dependent human calcium channels has been cloned herein by nucleic acid amplication of cDNA from selected tissues or by screening human cDNA libraries prepared from isolated poly A+ mRNA from cell lines or tissue of human origin having such calcium channels. Among the sources of such cells or tissue for obtaining mRNA are human brain tissue or a human cell line of neural origin, such as a neuroblastoma cell line, human skeletal muscle or smooth muscle cells, and the like. Methods of preparing cDNA libraries are well known in the art [see generally Ausubel *et al.* (1987) *Current*

Protocols in Molecular Biology, Wiley-Interscience, New York; and Davis *et al.* (1986) *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., New York].

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode transmembrane domains, sequences predicted to encode cytoplasmic loops, signal sequences, ligand-binding sites, and other functionally significant sequences (see Table, below). Either the full-length subunit-encoding DNA or fragments thereof can be used as probes, preferably labeled with suitable label means for ready detection. When fragments are used as probes, preferably the DNA sequences will be typically from the carboxyl-end-encoding portion of the DNA, and most preferably will include predicted transmembrane domain-encoding portions based on hydropathy analysis of the deduced amino acid sequence [see, e.g., Kyte and Doolittle [(1982) *J. Mol. Biol.* 167:105].

Riboprobes that specific for human calcium channel subunit types or subtypes have been prepared. These probes are useful for identifying expression of particular subunits in selected tissues and cells. The regions from which the probes were prepared were identified by comparing the DNA and amino acid sequences of all known α or β subunit subtypes. Regions of least homology, preferably human-derived sequences, and generally about 250 to about 600 nucleotides were selected. Numerous riboprobes for α and β subunits have been prepared; some of these are listed in the following Table.

TABLE 2
SUMMARY OF RNA PROBES

SUBUNIT SPECIFICITY	NUCLEOTIDE POSITION	PROBE NAME	PROBE TYPE	ORIENTATION
α1A generic	3357-3840	pGEM7Zα1A*	riboprobe	n/a
	761-790	SE700	oligo	antisense
	3440-3464	SE718	oligo	antisense
	3542-3565	SE724	oligo	sense
α1B generic	3091-3463	$pGEM7Z\alpha1B_{cyt}$	riboprobe	n/a
	6635-6858	$pGEM7Z\alpha1B_{cooh}$	riboprobe	n/a
α1B-1 specific	6490-6676	pCRII α1B-1/187	riboprobe	n/a
α1E generic	3114-3462	pGEM7Zα1E	riboprobe	n/a
α2b	1321-1603	pCRIIα2b	riboprobe	n/a
β generic(?)	212-236	SE300	oligo	antisense
β1 generic	1267-1291	SE301	oligo	antisense
β1-2 specific	1333-1362	SE17	oligo	antisense
	1682-1706	SE23	oligo	sense
	2742-2766	SE43	oligo	antisense
	27-56	SE208	oligo	antisense
	340-364	SE274	oligo	antisense
	340-364	SE275	oligo	sense
β3 specific	1309-1509		riboprobe	n/a
β4 specific	1228-1560		riboprobe	n/a

* The pGEM series are available from Promega, Madison WI; see also, U.S. Patent No. 4,766,072.

The above-noted nucleotide regions are also useful in selecting regions of the protein for preparation of subunit-specific antibodies, discussed below.

The DNA clones and fragments thereof provided herein thus can be used to isolate genomic clones encoding each subunit and to isolate any splice variants by hybridization screening of libraries prepared from different human tissues. Nucleic acid amplification techniques, which are well known in the art, can also be used to locate DNA encoding splice variants

of human calcium channel subunits. This is accomplished by employing oligonucleotides based on DNA sequences surrounding divergent sequence(s) as primers for amplifying human RNA or genomic DNA. Size and sequence determinations of the amplification products can reveal the existence of splice variants. Furthermore, isolation of human genomic DNA sequences by hybridization can yield DNA containing multiple exons, separated by introns, that correspond to different splice variants of transcripts encoding human calcium channel subunits.

Once DNA encoding a calcium channel subunit is isolated, ribonuclease (RNase) protection assays can be employed to determine which tissues express mRNA encoding a particular calcium channel subunit or variant. These assays provide a sensitive means for detecting and quantitating an RNA species in a complex mixture of total cellular RNA. The subunit DNA is labeled and hybridized with cellular RNA. If complementary mRNA is present in the cellular RNA, a DNA-RNA hybrid results. The RNA sample is then treated with RNase, which degrades single-stranded RNA. Any RNA-DNA hybrids are protected from RNase degradation and can be visualized by gel electrophoresis and autoradiography. *In situ* hybridization techniques can also be used to determine which tissues express mRNA encoding a particular calcium channel subunit. The labeled subunit DNAs are hybridized to different tissue slices to visualize subunit mRNA expression.

With respect to each of the respective subunits (α_1, α_2, β or γ) of human calcium channels, once the DNA encoding the channel subunit was identified by a nucleic acid screening method, the isolated clone was used for further screening to identify overlapping clones. Some of the cloned DNA fragments can and have been subcloned into an appropriate vector such as pIBI24/25 (IBI, New Haven, CT), M13mp18/19, pGEM4, pGEM3, pGEM7Z, pSP72 and other such vectors known to those of skill in this art, and characterized by DNA sequencing and restriction enzyme mapping. A sequential series of

overlapping clones may thus be generated for each of the subunits until a full-length clone can be prepared by methods, known to those of skill in the art, that include identification of translation initiation (start) and translation termination (stop) codons. For expression of the cloned DNA, the 5′ noncoding region and other transcriptional and translational control regions of such a clone may be replaced with an efficient ribosome binding site and other regulatory regions as known in the art. Other modifications of the 5′ end, known to those of skill in the art, that may be required to optimize translation and/or transcription efficiency may also be effected, if deemed necessary.

Examples II-VIIII, below, describe in detail the cloning of each of the various subunits of a human calcium channel as well as subtypes and splice variants, including tissue-specific variants thereof. In the few instances in which partial sequences of a subunit are disclosed, it is well within the skill of the art, in view of the teaching herein, to obtain the corresponding full-length clones and sequence thereof encoding the subunit, subtype or splice variant thereof using the methods described above and exemplified below.

Identification and isolation of DNA encoding α_1 subunits

A number of voltage-dependent calcium channel α_1 subunit genes, which are expressed in the human CNS and in other tissues, have been identified and have been designated as α_{1A}, α_{1B} (or VDCC IV), α_{1C} (or VDCC II), α_{1D} (or VDCC III) and α_{1E}. DNA, isolated from a human neural cDNA library, that encodes each of the subunit types has been isolated. DNA encoding subtypes of each of the types, which arise as splice variants are also provided. Subtypes are herein designated, for example, as α_{1B-1}, α_{1B-2}.

The α_1 subunits types A B, C, D and E of voltage-dependent calcium channels, and subtypes thereof, differ with respect to sensitivity to known classes of calcium channel

agonists and antagonists, such as DHPs, phenylalkylamines, omega conotoxin (ω-CgTx), the funnel web spider toxin ω-Aga-IV, and pyrazonoylguanidines. These subunit types also appear to differ in the holding potential and in the kinetics of currents produced upon depolarization of cell membranes containing calcium channels that include different types of α_1 subunits.

DNA that encodes an α_1 subunit that binds to at least one compound selected from among dihydropyridines, phenylalkylamines, ω-CgTx, components of funnel web spider toxin, and pyrazonoylguanidines is provided. For example, the α_{1B} subunit provided herein appears to specifically interact with ω-CgTx in N-type channels, and the α_{1D} subunit provided herein specifically interacts with DHPs in L-type channels.

Identification and isolation of DNA encoding the α_{1D} human calcium channel subunit

The α_{1D} subunit cDNA has been isolated using fragments of the rabbit skeletal muscle calcium channel α_1 subunit cDNA as a probe to screen a cDNA library of a human neuroblastoma cell line, IMR32, to obtain clone α1.36. This clone was used as a probe to screen additional IMR32 cell cDNA libraries to obtain overlapping clones, which were then employed for screening until a sufficient series of clones to span the length of the nucleotide sequence encoding the human α_{1D} subunit was obtained. Full-length clones encoding α_{1D} were constructed by ligating portions of partial α_{1D} clones as described in Example II. SEQ ID No. 1 shows the 7,635 nucleotide sequence of the cDNA encoding the α_{1D} subunit. There is a 6,483 nucleotide sequence reading frame which encodes a sequence of 2,161 amino acids (as set forth in SEQ ID No. 1).

SEQ ID No. 2 provides the sequence of an alternative exon encoding the IS6 transmembrane domain [see Tanabe, T., *et al.* (1987) *Nature 328*:313-318 for a description of transmembrane domain terminology] of the α_{1D} subunit.

SEQ ID No. 1 also shows the 2,161 amino acid sequence deduced from the human neuronal calcium channel α_{1D} subunit

DNA. Based on the amino acid sequence, the α_{1D} protein has a calculated Mr of 245,163. The α_{1D} subunit of the calcium channel contains four putative internal repeated sequence regions. Four internally repeated regions represent 24 putative transmembrane segments, and the amino- and carboxyl-termini extend intracellularly.

The α_{1D} subunit has been shown to mediate DHP-sensitive, high-voltage-activated, long-lasting calcium channel activity. This calcium channel activity was detected when oöcytes were co-injected with RNA transcripts encoding an α_{1D} and β_{1-2} or α_{1D}, α_{2b} and β_{1-2} subunits. This activity was distinguished from Ba^{2+} currents detected when oöcytes were injected with RNA transcripts encoding the $\beta_{1-2} \pm \alpha_{2b}$ subunits. These currents pharmacologically and biophysically resembled Ca^{2+} currents reported for uninjected oöcytes.

Identification and isolation of DNA encoding the α_{1A} human calcium channel subunit

Biological material containing DNA encoding a portion of the α_{1A} subunit had been deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

A portion of an α_{1A} subunit is encoded by an approximately 3 kb insert in λgt10 phage designated α1.254 in *E. coli* host strain NM514. A phage lysate of this material has been deposited as at the American Type Culture Collection under

ATCC Accession No. 75293, as described above. DNA encoding α_{1A} may also be identified by screening with a probe prepared from DNA that has SEQ ID No. 21:

5' CTCAGTACCATCTCTGATACCAGCCCCA 3'.

α_{1A} splice variants have been obtained. The sequences of two α_{1A} splice variants, α_{1a-1} and α_{1a-2} are set forth in SEQ. ID Nos. 22 and 23. Other splice variants may be obtained by screening a human library as described above or using all or a portion of the sequences set forth in SEQ ID Nos. 22 and 23.

Identification and isolation of DNA encoding the α_{1B} human calcium channel subunit

DNA encoding the α_{1B} subunit was isolated by screening a human basal ganglia cDNA library with fragments of the rabbit skeletal muscle calcium channel α_1 subunit-encoding cDNA. A portion of one of the positive clones was used to screen an IMR32 cell cDNA library. Clones that hybridized to the basal ganglia DNA probe were used to further screen an IMR32 cell cDNA library to identify overlapping clones that in turn were used to screen a human hippocampus cDNA library. In this way, a sufficient series of clones to span nearly the entire length of the nucleotide sequence encoding the human α_{1B} subunit was obtained. Nucleic acid amplification of specific regions of the IMR32 cell α_{1B} mRNA yielded additional segments of the α_{1B} coding sequence.

A full-length α_{1B} DNA clone was constructed by ligating portions of the partial cDNA clones as described in Example II.C. SEQ ID Nos. 7 and 8 show the nucleotide sequences of DNA clones encoding the α_{1B} subunit as well as the deduced amino acid sequences. The α_{1B} subunit encoded by SEQ ID No. 7 is referred to as the α_{1B-1} subunit to distinguish it from another α_{1B} subunit, α_{1B-2}, encoded by the nucleotide sequence shown as SEQ ID No. 8, which is derived from alternative splicing of the α_{1B} subunit transcript.

Nucleic acid amplification of IMR32 cell mRNA using oligonucleotide primers designed according to nucleotide

sequences within the α_{1B-1}-encoding DNA has identified variants of the α_{1B} transcript that appear to be splice variants because they contain divergent coding sequences.

Identification and isolation of DNA encoding the α_{1C} human calcium channel subunit

Numerous α_{1C}-specific DNA clones were isolated. Characterization of the sequence revealed the α_{1C} coding sequence, the α_{1C} initiation of translation sequence, and an alternatively spliced region of α_{1C}. Alternatively spliced variants of the α_{1C} subunit have been identified. SEQ ID No. 3 sets forth DNA encoding a substantial protion of an α_{1C} subunit. The DNA sequences set forth in SEQ ID No. 4 and No. 5 encode two possible amino terminal ends of the α_{1C} protein. SEQ ID No. 6 encodes an alternative exon for the IV S3 transmembrane domain. The sequences of substantial portions of two α_{1C} splice variants, designated α_{1C-1} and α_{1C-2}, are set forth in SEQ ID NOs. 3 and 36, respectively.

The isolation and identification of DNA clones encoding portions of the α_{1C} subunit is described in detail in Example II.

Identification and isolation of DNA encoding the α_{1E} human calcium channel subunit

DNA encoding α_{1E} human calcium channel subunits have been isolated from an oligo dT-primed human hippocampus library. The resulting clones, which are splice variants, were designated α_{1E-1} and α_{1E-3}. The subunit designated α_{1E-1} has the amino acid sequence set forth in SEQ ID No. 24, and a subunit designated α_{1E-3} has the amino acid sequence set forth in SEQ ID No. 25. These splice variants differ by virtue of a 57 base pair insert between nucleotides 2405 and 2406 of SEQ. ID No. 24.

The α_{1E} subunits provided herein appear to participate in the formation of calcium channels that have properties of high-voltage activated calcium channels and low-voltage activated channels. These channels are rapidly inactivating

compared to other high voltage-activated calcium channels. In addition these channels exhibit pharmacological profiles that are similar to voltage-activated channels, but are also sensitive to DHPs and ω-Aga-IVA, which block certain high voltage activated channels. Additional details regarding the electrophysiology and pharmacology of channels containing α_{1E} subunits is provided in Example VII. F.

Identification and isolation of DNA encoding encoding additional α_1 human calcium channel subunit types and subtypes

DNA encoding additional α_1 subunits can be isolated and identified using the DNA provided herein as described for the α_{1A}, α_{1B}, α_{1C}, α_{1D} and α_{1E} subunits or using other methods known to those of skill in the art. In particular, the DNA provided herein may be used to screen appropriate libraries to isolate related DNA. Full-length clones can be constructed using methods, such as those described herein, and the resulting subunits characterized by comparison of their sequences and electrophysiological and pharmacological properties with the subunits exemplified herein.

Identification and isolation of DNA encoding β human calcium channel subunits

DNA encoding β_1

To isolate DNA encoding the β_1 subunit, a human hippocampus cDNA library was screened by hybridization to a DNA fragment encoding a rabbit skeletal muscle calcium channel β subunit. A hybridizing clone was selected and was in turn used to isolate overlapping clones until the overlapping clones encompassing DNA encoding the entire the human calcium channel β subunit were isolated and sequenced.

Five alternatively spliced forms of the human calcium channel β_1 subunit have been identified and DNA encoding a number of forms have been isolated. These forms are designated β_{1-1}, expressed in skeletal muscle, β_{1-2}, expressed in the CNS, β_{1-3}, also expressed in the in the CNS, β_{1-4}, expressed in aorta tissue and HEK 293 cells, and β_{1-5},

expressed in HEK 293 cells. Full-length DNA clones encoding the β_{1-2} and β_{1-3} subunits have been constructed. The subunits β_{1-1}, β_{1-2}, β_{1-4} and β_{1-5} have been identified by nucleic acid amplification analysis as alternatively spliced forms of the β subunit. Sequences of the β_1 splice variants are set forth in SEQ ID Nos. 9, 10 and 33-35.

DNA encoding β_2

DNA encoding the β_2 splice variants has been obtained. These splice variants include β_{2C}-β_{2E}. Splice variants β_{2C}-β_{2E} include all of sequence set forth in SEQ ID No. 26, except for the portion at the 5' end (up to nucleotide 182), which differs among splice variants. The sequence set forth in SEQ ID No. 26 encodes β_{2D}. Additional splice variants may be isolated using the methods described herein and oligonucleotides including all or portions of the DNA set forth in SEQ ID. No. 26 or may be prepared or obtained as described in the Examples. The sequences of β_2 splice variants β_{2C} and β_{2E} are set forth in SEQ ID Nos. 37 and 38, respectively.

DNA encoding β_3

DNA encoding the β_3 subunit and any splice variants thereof may be isolated by screening a library, as described above for the β_1 subunit, using DNA probes prepared according to SEQ ID Nos. 19, 20 or using all or a portion of the deposited β_3 clone plasmid β1.42 (ATCC Accession No. 69048).

The *E. coli* host containing plasmid β1.42 that includes DNA encoding a β_3 subunit has been deposited as ATCC Accession No. 69048 in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A. under the terms of the Budapest Treaty on the International Recognition of Deposits of Microorganisms for Purposes of Patent Procedure and the Regulations promulgated under this Treaty. Samples of the deposited material are and will be available to industrial property offices and other persons legally entitled to receive them under the terms of the Treaty and Regulations and otherwise in compliance with the patent laws and regulations

of the United States of America and all other nations or international organizations in which this application, or an application claiming priority of this application, is filed or in which any patent granted on any such application is granted.

The β_3 encoding plasmid is designated β1.42. The plasmid contains a 2.5 kb *Eco*RI fragment encoding β_3 inserted into vector pGem®7zF(+) and has been deposited in *E. coli* host strain DH5α. The sequences of β_3 splice variants, designated β_{3-1} and β_{3-2} are set forth in SEQ ID Nos. 19 and 20, respectively.

Identification and isolation of DNA encoding the α2 human calcium channel subunit

DNA encoding a human neuronal calcium channel α_2 subunit was isolated in a manner substantially similar to that used for isolating DNA encoding an α_1 subunit, except that a human genomic DNA library was probed under low and high stringency conditions with a fragment of DNA encoding the rabbit skeletal muscle calcium channel α_2 subunit. The fragment included nucleotides having a sequence corresponding to the nucleotide sequence between nucleotides 43 and 272 inclusive of rabbit back skeletal muscle calcium channel α_2 subunit cDNA as disclosed in PCT International Patent Application Publication No. WO 89/09834, which corresponds to U.S. Application Serial No. 07/620,520 (now allowed U.S. Application Serial No. 07/914,231), which is a continuation-in-part of United States Serial No. 176,899, filed April 4, 1988.

Example IV describes the isolation of DNA clones encoding α_2 subunits of a human calcium channel from a human DNA library using genomic DNA and cDNA clones, identified by hybridization to the genomic DNA, as probes.

SEQ ID Nos. 11 and 29-32 show the sequence of DNA encoding α_2 subunits. As described in Example V, nucleic acid amplification analysis of RNA from human skeletal muscle, brain tissue and aorta using oligonucleotide primers specific for a region of the human neuronal α_2 subunit cDNA that

diverges from the rabbit skeletal muscle calcium channel α_2 subunit cDNA identified splice variants of the human calcium channel α_2 subunit transcript.

Identification and isolation of DNA encoding γ human calcium channel subunits

DNA encoding a portion of a human neuronal calcium channel γ subunit has been isolated as described in detail in Example VI. SEQ ID No. 14 shows the nucleotide sequence at the 3'-end of this DNA which includes a reading frame encoding a sequence of 43 amino acid residues. Since the portion that has been obtained is homologous to the rabbit clone, described in allowed co-owned U.S. Application Serial No. 07/482,384, the remainder of the clone can be obtained using routine methods.

Antibodies

Antibodies, monoclonal or polyclonal, specific for calcium channel subunit subtypes or for calcium channel types can be prepared employing standard techniques, known to those of skill in the art, using the subunit proteins or portions thereof as antigens. Anti-peptide and anti-fusion protein antibodies can be used [see, for example, Bahouth et al. (1991) *Trends Pharmacol. Sci.* 12:338-343; *Current Protocols in Molecular Biology* (Ausubel et al., eds.) John Wiley and Sons, New York (1984)]. Factors to consider in selecting portions of the calcium channel subunits for use as immunogens (as either a synthetic peptide or a recombinantly produced bacterial fusion protein) include antigenicity accessibility (i.e., extracellular and cytoplasmic domains), uniqueness to the particular subunit, and other factors known to those of skill in this art.

The availability of subunit-specific antibodies makes possible the application of the technique of immunohistochemistry to monitor the distribution and expression density of various subunits (e.g., in normal vs diseased brain tissue). Such antibodies could also be employed in diagnostic, such as LES diagnosis, and therapeutic

applications, such as using antibodies that modulate activities of calcium channels.

The antibodies can be administered to a subject employing standard methods, such as, for example, by intraperitoneal, intramuscular, intravenous, or subcutaneous injection, implant or transdermal modes of administration, and the like. One of skill in the art can empirically determine dose forms, treatment regiments, etc., depending on the mode of administration employed.

Subunit-specific monoclonal antibodies and polyclonal antisera have been prepared. The regions from which the antigens were identified by comparing the DNA and amino acid sequences of all known α or β subunit subtypes. Regions of least homology, preferably human-derived sequences were selected. The selected regions or fusion proteins containing the selected regions are used as immunogens. Hydrophobicity analyses of residues in selected protein regions and fusion proteins are also performed; regions of high hydrophobicity are avoided. Also, and more importantly, when preparing fusion proteins in bacterial hosts, rare codons are avoided. In particular, inclusion of 3 or more successive rare codons in a selected host is avoided. Numerous antibodies, polyclonal and monoclonal, specific for α or β subunits types or subtypes have been prepared; some of these are listed in the following Table. Exemplary antibodies and peptide antigens used to prepare the antibodies are set forth in the following Table:

TABLE 3

SPECIFICITY	AMINO ACID NUMBER	ANTIGEN NAME	ANTIBODY TYPE
α1 generic	112-140	peptide 1A#1	polyclonal
α1 generic	1420-1447	peptide 1A#2	polyclonal
α1A generic	1048-1208	α1A#2(b)GST fusion*	polyclonal
			monoclonal
α1B generic	983-1106	α1B#2(b) GST fusion	polyclonal
			monoclonal

α1B-1	2164-2339	α1B-1#3 GST fusion	polyclonal
α1B-2	2164-2237	α1B-2#4 GST fusion	polyclonal
α1E generic	985-1004 (α1E-3)	α1E#2(a) GST fusion	polyclonal

* GST gene fusion system is available from Pharmacia; see also, Smith et al. (1988) Gene 67:31. The system provides pGEX plasmids that are designed for inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. Upon expression in a bacterial host, the resulting fusion proteins are purified from bacterial lysates by affinity chromatography.

The GST fusion proteins are each specific for the cytoplasmic loop region IIS6-IIS1, which is a region of low subtype homology for all subtypes, including α_{1C} and α_{1D}, for which similar fusions and antisera can be prepared.

Preparation of recombinant eukaryotic cells containing DNA encoding heterologous calcium channel subunits

DNA encoding one or more of the calcium channel subunits or a portion of a calcium channel subunit may be introduced into a host cell for expression or replication of the DNA. Such DNA may be introduced using methods described in the following examples or using other procedures well known to those skilled in the art. Incorporation of cloned DNA into a suitable expression vector, transfection of eukaryotic cells with a plasmid vector or a combination of plasmid vectors, each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are also well known in the art [see, *e.g.*, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press]. Cloned full-length DNA encoding any of the subunits of a human calcium channel may be introduced into a plasmid vector for expression in a eukaryotic cell. Such DNA may be genomic DNA or cDNA. Host cells may be transfected with one or a combination of the plasmids, each of which encodes at least one calcium channel subunit. Alternatively, host cells may be transfected with linear DNA using methods well known to those of skill in the art.

While the DNA provided herein may be expressed in any eukaryotic cell, including yeast cells such as *P. pastoris*

[see, *e.g.*, Cregg *et al.* (1987) *Bio/Technology* 5:479], mammalian expression systems for expression of the DNA encoding the human calcium channel subunits provided herein are preferred.

The heterologous DNA may be introduced by any method known to those of skill in the art, such as transfection with a vector encoding the heterologous DNA. Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCDNA1, or pcDNA-amp and MMTV promoter-based vectors. DNA encoding the human calcium channel subunits has been inserted in the vector pCDNA1 at a position immediately following the CMV promoter. The vector pCDNA1 is presently preferred.

Stably or transiently transfected mammalian cells may be prepared by methods known in the art by transfecting cells with an expression vector having a selectable marker gene such as the gene for thymidine kinase, dihydrofolate reductase, neomycin resistance or the like, and, for transient transfection, growing the transfected cells under conditions selective for cells expressing the marker gene. Functional voltage-dependent calcium channels have been produced in HEK 293 cells transfected with a derivative of the vector pCDNA1 that contains DNA encoding a human calcium channel subunit.

The heterologous DNA may be maintained in the cell as an episomal element or may be integrated into chromosomal DNA of the cell. The resulting recombinant cells may then be cultured or subcultured (or passaged, in the case of mammalian cells) from such a culture or a subculture thereof. Methods for transfection, injection and culturing recombinant cells are known to the skilled artisan. Eukaryotic cells in which DNA or RNA may be introduced, include any cells that are transfectable by such DNA or RNA or into which such DNA may be injected. Virtually any eukaryotic cell can serve as a

vehicle for heterologous DNA. Preferred cells are those that can also express the DNA and RNA and most preferred cells are those that can form recombinant or heterologous calcium channels that include one or more subunits encoded by the heterologous DNA. Such cells may be identified empirically or selected from among those known to be readily transfected or injected. Preferred cells for introducing DNA include those that can be transiently or stably transfected and include, but are not limited to, cells of mammalian origin, such as COS cells, mouse L cells, CHO cells, human embryonic kidney cells, African green monkey cells and other such cells known to those of skill in the art, amphibian cells, such as *Xenopus laevis* oöcytes, or those of yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris*. Preferred cells for expressing injected RNA transcripts or cDNA include *Xenopus laevis* oöcytes. Cells that are preferred for transfection of DNA are those that can be readily and efficiently transfected. Such cells are known to those of skill in the art or may be empirically identified. Preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that can be frozen in liquid nitrogen and then thawed and regrown. Such HEK 293 cells are described, for example in U.S. Patent No. 5,024,939 to Gorman [see, also Stillman *et al.* (1985) *Mol. Cell.Biol.* 5:2051-2060].

The cells may be used as vehicles for replicating heterologous DNA introduced therein or for expressing the heterologous DNA introduced therein. In certain embodiments, the cells are used as vehicles for expressing the heterologous DNA as a means to produce substantially pure human calcium channel subunits or heterologous calcium channels. Host cells containing the heterologous DNA may be cultured under conditions whereby the calcium channels are expressed. The calcium channel subunits may be purified using protein purification methods known to those of skill in the art. For example, antibodies, such as those provided herein, that specifically bind to one or more of the subunits may be used

for affinity purification of the subunit or calcium channels containing the subunits.

Substantially pure subunits of a human calcium channel α_1 subunits of a human calcium channel, α_2 subunits of a human calcium channel, β subunits of a human calcium channel and γ subunits of a human calcium channel are provided. Substantially pure isolated calcium channels that contain at least one of the human calcium channel subunits are also provided. Substantially pure calcium channels that contain a mixture of one or more subunits encoded by the host cell and one or more subunits encoded by heterologous DNA or RNA that has been introduced into the cell are also provided. Substantially pure subtype- or tissue-type specific calcium channels are also provided.

In other embodiments, eukaryotic cells that contain heterologous DNA encoding at least one of an α_1 subunit of a human calcium channel , an α_2 subunit of a human calcium channel, a β subunit of a human calcium channel and a γ subunit of a human calcium channel are provided. In accordance with one preferred embodiment, the heterologous DNA is expressed in the eukaryotic cell and preferably encodes a human calcium channel α_1 subunit.

Expression of heterologous calcium channels: electrophysiology and pharmacology

Electrophysiological methods for measuring calcium channel activity are kwown to those of skill in the art and are exemplified herein. Any such methods may be used in order to detect the formation of functional calcium channels and to characterize the kinetics and other characteristics of the resulting currents. Pharmacological studies may be combined with the electrophysiological measurements in order to further characterize the calcium channels.

With respect to measurement of the activity of functional heterologous calcium channels, preferably, endogenous ion channel activity and, if desired, heterologous channel activity of channels that do not contain the desired subunits,

of a host cell can be inhibited to a significant extent by chemical, pharmacological and electrophysiological means, including the use of differential holding potential, to increase the S/N ratio of the measured heterologous calcium channel activity.

Thus, various combinations of subunits encoded by the DNA provided herein are introduced into eukaryotic cells. The resulting cells can be examined to ascertain whether functional channels are expressed and to determine the properties of the channels. In particularly preferred aspects, the eukaryotic cell which contains the heterologous DNA expresses it and forms a recombinant functional calcium channel activity. In more preferred aspects, the recombinant calcium channel activity is readily detectable because it is a type that is absent from the untransfected host cell or is of a magnitude and/or pharmacological properties or exhibits biophysical properties not exhibited in the untransfected cell.

The eukaryotic cells can be transfected with various combinations of the subunit subtypes provided herein. The resulting cells will provide a uniform population of calcium channels for study of calcium channel activity and for use in the drug screening assays provided herein. Experiments that have been performed have demonstrated the inadequacy of prior classification schemes.

Preferred among transfected cells is a recombinant eukaryotic cell with a functional heterologous calcium channel. The recombinant cell can be produced by introduction of and expression of heterologous DNA or RNA transcripts encoding an α_1 subunit of a human calcium channel, more preferably also expressing, a heterologous DNA encoding a β subunit of a human calcium channel and/or heterologous DNA encoding an α_2 subunit of a human calcium channel. Especially preferred is the expression in such a recombinant cell of each of the α_1, β and α_2 subunits encoded by such heterologous DNA or RNA transcripts, and optionally expression of heterologous

DNA or an RNA transcript encoding a γ subunit of a human calcium channel. The functional calcium channels may preferably include at least an α_1 subunit and a β subunit of a human calcium channel. Eukaryotic cells expressing these two subunits and also cells expressing additional subunits, have been prepared by transfection of DNA and by injection of RNA transcripts. Such cells have exhibited voltage-dependent calcium channel activity attributable to calcium channels that contain one or more of the heterologous human calcium channel subunits. For example, eukaryotic cells expressing heterologous calcium channels containing an α_2 subunit in addition to the α_1 subunit and a β subunit have been shown to exhibit increased calcium selective ion flow across the cellular membrane in response to depolarization, indicating that the α_2 subunit may potentiate calcium channel function. Cells that have been co-transfected with increasing ratios of α_2 to α_1 and the activity of the resulting calcium channels has been measured. The results indicate that α_2 increasing the amount of α_2-encoding DNA relative to the other transfected subunits increases calcium channel activity.

Eukaryotic cells which express heterologous calcium channels containing at least a human α_1 subunit, a human β subunit and a human α_2 subunit are preferred. Eukaryotic cells transformed with a composition containing cDNA or an RNA transcript that encodes an α_1 subunit alone or in combination with a β and/or an α_2 subunit may be used to produce cells that express functional calcium channels. Since recombinant cells expressing human calcium channels containing all of the human subunits encoded by the heterologous cDNA or RNA are especially preferred, it is desirable to inject or transfect such host cells with a sufficient concentration of the subunit-encoding nucleic acids to form calcium channels that contain the human subunits encoded by heterologous DNA or RNA. The precise amounts and ratios of DNA or RNA encoding the subunits may be empirically determined and optimized for a

particular combination of subunits, cells and assay conditions.

In particular, mammalian cells have been transiently and stably tranfected with DNA encoding one or more human calcium channel subunits. Such cells express heterologous calcium channels that exhibit pharmacological and electrophysiological properties that can be ascribed to human calcium channels. Such cells, however, represent homogeneous populations and the pharmacological and electrophysiological data provides insights into human calcium channel activity heretofore unattainable. For example, HEK cells that have been transiently transfected with DNA encoding the α_{1E-1}, α_{2b}, and β_{1-3} subunits. The resulting cells transiently express these subunits, which form calcium channels that have properties that appear to be a pharmacologically distinct class of voltage-activated calcium channels distinct from those of L-, N-, T- and P-type channels. The observed α_{1E} currents were insensitive to drugs and toxins previously used to define other classes of voltage-activated calcium channels.

HEK cells that have been transfiently transfected with DNA encoding α_{1B-1}, α_{2b}, and β_{1-2} express heterologous calcium channels that exhibt sensitivity to ω-conotoxin and currents typical of N-type channels. It has been found that alteration of the molar raios of α_{1B-1}, α_{2b} and β_{1-2} introduced into the cells into to achieve equivalent mRNA levels significantly increased the number of receptors per cell, the current density, and affected the K_d for ω-conotoxin.

The electrophyiological properties of these channels produced from α_{1B-1}, α_{2b}, and β_{1-2} was compared with those of channels produced by transiently transfecting HEK cells with DNA encoding α_{1B-1}, α_{2b} and β_{1-3}. The channels exhibited similar voltage dependence of activation, substantially identical voltage dependence, similar kinetics of activation and tail currents that could be fit by a single exponential. The voltage dependence of the kinetics of inactivation was significantly different at all voltages examined.

In certain embodiments, the eukaryotic cell with a heterologous calcium channel is produced by introducing into the cell a first composition, which contains at least one RNA transcript that is translated in the cell into a subunit of a human calcium channel. In preferred embodiments, the subunits that are translated include an α_1 subunit of a human calcium channel. More preferably, the composition that is introduced contains an RNA transcript which encodes an α_1 subunit of a human calcium channel and also contains (1) an RNA transcript which encodes a β subunit of a human calcium channel and/or (2) an RNA transcript which encodes an α_2 subunit of a human calcium channel. Especially preferred is the introduction of RNA encoding an α_1, a β and an α_2 human calcium channel subunit, and, optionally, a γ subunit of a human calcium channel. Methods for *in vitro* transcription of a cloned DNA and injection of the resulting RNA into eukaryotic cells are well known in the art. Transcripts of any of the full-length DNA encoding any of the subunits of a human calcium channel may be injected alone or in combination with other transcripts into eukaryotic cells for expression in the cells. Amphibian oöcytes are particularly preferred for expression of *in vitro* transcripts of the human calcium channel subunit cDNA clones provided herein. Amphibian oocytes that express functional heterologous calcium channels have been produced by this method.

Assays and Clinical uses of the cells and calcium channels

Assays

Assays for identifying compounds that modulate calcium channel activity

Among the uses for eukaryotic cells which recombinantly express one or more subunits are assays for determining whether a test compound has calcium channel agonist or antagonist activity. These eukaryotic cells may also be used to select from among known calcium channel agonists and antagonists those exhibiting a particular calcium channel

subtype specificity and to thereby select compounds that have potential as disease- or tissue-specific therapeutic agents.

In vitro methods for identifying compounds, such as calcium channel agonist and antagonists, that modulate the activity of calcium channels using eukaryotic cells that express heterologous human calcium channels are provided.

In particular, the assays use eukaryotic cells that express heterologous human calcium channel subunits encoded by heterologous DNA provided herein, for screening potential calcium channel agonists and antagonists which are specific for human calcium channels and particularly for screening for compounds that are specific for particular human calcium channel subtypes. Such assays may be used in conjunction with methods of rational drug design to select among agonists and antagonists, which differ slightly in structure, those particularly useful for modulating the activity of human calcium channels, and to design or select compounds that exhibit subtype- or tissue- specific calcium channel antagonist and agonist activities. These assays should accurately predict the relative therapeutic efficacy of a compound for the treatment of certain disorders in humans. In addition, since subtype-and tissue-specific calcium channel subunits are provided, cells with tissue- specific or subtype-specific recombinant calcium channels may be prepared and used in assays for identification of human calcium channel tissue- or subtype-specific drugs.

Desirably, the host cell for the expression of calcium channel subunits does not produce endogenous calcium channel subunits of the type or in an amount that substantially interferes with the detection of heterologous calcium channel subunits in ligand binding assays or detection of heterologous calcium channel function, such as generation of calcium current, in functional assays. Also, the host cells preferably should not produce endogenous calcium channels which detectably interact with compounds having, at physiological concentrations (generally nanomolar or picomolar

concentrations), affinity for calcium channels that contain one or all of the human calcium channel subunits provided herein.

With respect to ligand binding assays for identifying a compound which has affinity for calcium channels, cells are employed which express, preferably, at least a heterologous α_1 subunit. Transfected eukaryotic cells which express at least an α_1 subunit may be used to determine the ability of a test compound to specifically bind to heterologous calcium channels by, for example, evaluating the ability of the test compound to inhibit the interaction of a labeled compound known to specifically interact with calcium channels. Such ligand binding assays may be performed on intact transfected cells or membranes prepared therefrom.

The capacity of a test compound to bind to or otherwise interact with membranes that contain heterologous calcium channels or subunits thereof may be determined by using any appropriate method, such as competitive binding analysis, such as Scatchard plots, in which the binding capacity of such membranes is determined in the presence and absence of one or more concentrations of a compound having known affinity for the calcium channel. Where necessary, the results may be compared to a control experiment designed in accordance with methods known to those of skill in the art. For example, as a negative control, the results may be compared to those of assays of an identically treated membrane preparation from host cells which have not been transfected with one or more subunit-encoding nucleic acids.

The assays involve contacting the cell membrane of a recombinant eukaryotic cell which expresses at least one subunit of a human calcium channel, preferably at least an α_1 subunit of a human calcium channel, with a test compound and measuring the ability of the test compound to specifically bind to the membrane or alter or modulate the activity of a heterologous calcium channel on the membrane.

In preferred embodiments, the assay uses a recombinant cell that has a calcium channel containing an α_1 subunit of a human calcium channel in combination with a β subunit of a human calcium channel and/or an α_2 subunit of a human calcium channel. Recombinant cells expressing heterologous calcium channels containing each of the α_1, β and α_2 human subunits, and, optionally, a γ subunit of a human calcium channel are especially preferred for use in such assays.

In certain embodiments, the assays for identifying compounds that modulate calcium channel activity are practiced by measuring the calcium channel activity of a eukaryotic cell having a heterologous, functional calcium channel when such cell is exposed to a solution containing the test compound and a calcium channel-selective ion and comparing the measured calcium channel activity to the calcium channel activity of the same cell or a substantially identical control cell in a solution not containing the test compound. The cell is maintained in a solution having a concentration of calcium channel-selective ions sufficient to provide an inward current when the channels open. Rcombinant cells expressing calcium channels that include each of the α_1, β and α_2 human subunits, and, optionally, a γ subunit of a human calcium channel, are especially preferred for use in such assays. Methods for practicing such assays are known to those of skill in the art. For example, for similar methods applied with *Xenopus laevis* oöcytes and acetylcholine receptors, see, Mishina *et al.* [(1985) *Nature 313*:364] and, with such oöcytes and sodium channels [see, Noda *et al.* (1986) *Nature 322*:826-828]. For similar studies which have been carried out with the acetylcholine receptor, see, *e.g.*, Claudio *et al.* [(1987) *Science 238*:1688-1694].

Functional recombinant or heterologous calcium channels may be identified by any method known to those of skill in the art. For example, electrophysiological procedures for measuring the current across an ion-selective membrane of a cell, which are well known, may be used. The amount and

duration of the flow of calcium-selective ions through heterologous calcium channels of a recombinant cell containing DNA encoding one or more of the subunits provided herein has been measured using electrophysiological recordings using a two electrode and the whole-cell patch clamp techniques. In order to improve the sensitivity of the assays, known methods can be used to eliminate or reduce non-calcium currents and calcium currents resulting from endogenous calcium channels, when measuring calcium currents through recombinant channels. For example, the DHP Bay K 8644 specifically enhances L-type calcium channel function by increasing the duration of the open state of the channels [see, *e.g.*, Hess, J.B., *et al.* (1984) *Nature 311*:538-544]. Prolonged opening of the channels results in calcium currents of increased magnitude and duration. Tail currents can be observed upon repolarization of the cell membrane after activation of ion channels by a depolarizing voltage command. The opened channels require a finite time to close or "deactivate" upon repolarization, and the current that flows through the channels during this period is referred to as a tail current. Because Bay K 8644 prolongs opening events in calcium channels, it tends to prolong these tail currents and make them more pronounced.

In practicing these assays, stably or transiently transfected cells or injected cells that express voltage-dependent human calcium channels containing one or more of the subunits of a human calcium channel desirably may be used in assays to identify agents, such as calcium channel agonists and antagonists, that modulate calcium channel activity. Functionally testing the activity of test compounds, including compounds having unknown activity, for calcium channel agonist or antagonist activity to determine if the test compound potentiates, inhibits or otherwise alters the flow of calcium ions or other ions through a human calcium channel can be accomplished by (a) maintaining a eukaryotic cell which is transfected or injected to express a heterologous functional calcium channel capable of regulating the flow of calcium

channel-selective ions into the cell in a medium containing calcium channel-selective ions (i) in the presence of and (ii) in the absence of a test compound; (b) maintaining the cell under conditions such that the heterologous calcium channels are substantially closed and endogenous calcium channels of the cell are substantially inhibited (c) depolarizing the membrane of the cell maintained in step (b) to an extent and for an amount of time sufficient to cause (preferably, substantially only) the heterologous calcium channels to become permeable to the calcium channel-selective ions; and (d) comparing the amount and duration of current flow into the cell in the presence of the test compound to that of the current flow into the cell, or a substantially similar cell, in the absence of the test compound.

The assays thus use cells, provided herein, that express heterologous functional calcium channels and measure functionally, such as electrophysiologically, the ability of a test compound to potentiate, antagonize or otherwise modulate the magnitude and duration of the flow of calcium channel-selective ions, such as Ca^{++} or Ba^{++}, through the heterologous functional channel. The amount of current which flows through the recombinant calcium channels of a cell may be determined directly, such as electrophysiologically, or by monitoring an independent reaction which occurs intracellularly and which is directly influenced in a calcium (or other) ion dependent manner. Any method for assessing the activity of a calcium channel may be used in conjunction with the cells and assays provided herein. For example, in one embodiment of the method for testing a compound for its ability to modulate calcium channel activity, the amount of current is measured by its modulation of a reaction which is sensitive to calcium channel-selective ions and uses a eukaryotic cell which expresses a heterologous calcium channel and also contains a transcriptional control element operatively linked for expression to a structural gene that encodes an indicator protein. The transcriptional control

element used for transcription of the indicator gene is responsive in the cell to a calcium channel-selective ion, such as Ca^{2+} and Ba^{+}. The details of such transcriptional based assays are described in commonly owned PCT International Patent Application No. PCT/US91/5625, filed August 7, 1991, which claims priority to copending commonly owned allowed U.S. Application Serial No. 07/563,751, filed August 7, 1990; see also, commonly owned published PCT International Patent Application PCT US92/11090, which corresponds to co-pending U.S. Applications Serial Nos. 08/229,150 and 08/244,985.

Assays for diagnosis of LES

LES is an autoimmune disease characterized by an insufficient release of acetylcholine from motor nerve terminals which normally are responsive to nerve impulses. Immunoglobulins (IgG) from LES patients block individual voltage-dependent calcium channels and thus inhibit calcium channel activity [Kim and Neher, *Science 239*:405-408 (1988)]. A diagnostic assay for Lambert Eaton Syndrome (LES) is provided herein. The diagnostic assay for LES relies on the immunological reactivity of LES IgG with the human calcium channels or particular subunits alone or in combination or expressed on the surface of recombinant cells. For example, such an assay may be based on immunoprecipitation of LES IgG by the human calcium channel subunits and cells that express such subunits provided herein.

Clinical applications

In relation to therapeutic treatment of various disease states, the availability of DNA encoding human calcium channel subunits permits identification of any alterations in such genes (e.g., mutations) which may correlate with the occurrence of certain disease states. In addition, the creation of animal models of such disease states becomes possible, by specifically introducing such mutations into synthetic DNA fragments can then be introduced into laboratory animals or *in vitro* assay systems to determine the effects thereof.

Also, genetic screening can be carried out using the nucleotide sequences as probes. Thus, nucleic acid samples from subjects having pathological conditions suspected of involving alteration/modification of any one or more of the calcium channel subunits can be screened with appropriate probes to determine if any abnormalities exist with respect to any of the endogenous calcium channels. Similarly, subjects having a family history of disease states related to calcium channel dysfunction can be screened to determine if they are also predisposed to such disease states.

EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLE I: PREPARATION OF LIBRARIES USED FOR ISOLATION OF DNA ENCODING HUMAN NEURONAL VOLTAGE-DEPENDENT CALCIUM CHANNEL SUBUNITS

A. RNA Isolation

1. IMR32 cells

IMR32 cells were obtained from the American Type Culture Collection (ATCC Accession No. CCL127, Rockville, MD) and grown in DMEM, 10% fetal bovine serum, 1% penicillin/streptomycin (GIBCO, Grand Island, NY) plus 1.0 mM dibutyryl cAMP (dbcAMP) for ten days. Total RNA was isolated from the cells according to the procedure described by H.C. Birnboim [(1988) *Nucleic Acids Research 16*:1487-1497]. Poly(A^+) RNA was selected according to standard procedures [see, *e.g.*, Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press; pg. 7.26-7.29].

2. Human thalamus tissue

Human thalamus tissue (2.34 g), obtained from the National Neurological Research Bank, Los Angeles, CA, that had been stored frozen at -70°C was pulverized using a mortar and pestle in the presence of liquid nitrogen and the cells were lysed in 12 ml of lysis buffer (5 M guanidinium isothiocyanate, 50 mM TRIS, pH 7.4, 10 mM EDTA, 5% β-

mercaptoethanol). Lysis buffer was added to the lysate to yield a final volume of 17 ml. N-laurylsarcosine and CsCl were added to the mixture to yield final concentrations of 4% and 0.01 g/ml, respectively, in a final volume of 18 ml.

The sample was centrifuged at 9,000 rpm in a Sorvall SS34 rotor for 10 min at room temperature to remove the insoluble material as a pellet. The supernatant was divided into two equal portions and each was layered onto a 2-ml cushion of a solution of 5.7 M CsCl, 0.1 M EDTA contained in separate centrifuge tubes to yield approximately 9 ml per tube. The samples were centrifuged in an SW41 rotor at 37,000 rpm for 24 h at 20°C.

After centrifugation, each RNA pellet was resuspended in 3 ml ETS (10 mM TRIS, pH 7.4, 10 mM EDTA, 0.2% SDS) and combined into a single tube. The RNA was precipitated with 0.25 M NaCl and two volumes of 95% ethanol.

The precipitate was collected by centrifugation and resuspended in 4 ml PK buffer (0.05 M TRIS, pH 8.4, 0.14 M NaCl, 0.01 M EDTA, 1% SDS). Proteinase K was added to the sample to a final concentration of 200 μg/ml. The sample was incubated at 22°C for 1 h, followed by extraction with an equal volume of phenol:chloroform:isoamylalcohol (50:48:2) two times, followed by one extraction with an equal volume of chloroform: isoamylalcohol (24:1). The RNA was precipitated with ethanol and NaCl. The precipitate was resuspended in 400 μl of ETS buffer. The yield of total RNA was approximately 1.0 mg. Poly A$^+$ RNA (30 μg) was isolated from the total RNA according to standard methods as stated in Example I.A.1.

B. Library Construction

Double-stranded cDNA was synthesized according to standard methods [see, *e.g.*, Sambrook *et al.* (1989) IN: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8]. Each library was prepared in substantially the same manner except for differences in: 1) the oligonucleotide used to prime the first strand cDNA synthesis, 2) the adapters that were attached to the double-

stranded cDNA, 3) the method used to remove the free or unused adapters, and 4) the size of the fractionated cDNA ligated into the λ phage vector.

1. IMR32 cDNA library #1

Single-stranded cDNA was synthesized using IMR32 poly(A$^+$) RNA (Example I.A.1.) as a template and was primed using oligo $(dT)_{12-18}$ (Collaborative Research Inc., Bedford, MA). The single-stranded cDNA was converted to double-stranded cDNA and the yield was approximately 2μg. *Eco*I adapters:

5′-AATTCGGTACGTACACTCGAGC-3′ = 22-mer (SEQ ID No.15)

3′- GCCATGCATGTGAGCTCG-5′ = 18-mer (SEQ ID No.16)

also containing *Sna*BI and *Xho*I restriction sites were then added to the double-stranded cDNA according to the following procedure.

a. Phosphorylation of 18-mer

The 18-mer was phosphorylated using standard methods [see, *e.g.*, Sambrook *et al.* (1989) IN: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8] by combining in a 10 μl total volume the 18-mer (225 pmoles) with [^{32}P]γ-ATP (7000 Ci/mmole; 1.0 μl) and kinase (2 U) and incubating at 37° C for 15 minutes. After incubation, 1 μl 10 mM ATP and an additional 2 U of kinase were added and incubated at 37°C for 15 minutes.
Kinase was then inactivated by boiling for 10 minutes.

b. Hybridization of 22-mer

The 22-mer was hybridized to the phosphorylated 18-mer by addition of 225 pmoles of the 22-mer (plus water to bring volume to 15 μl), and incubation at 65°C for 5 minutes. The reaction was then allowed to slow cool to room temperature.

The adapters were thus present at a concentration of 15 pmoles/μl, and were ready for cDNA-adapter ligation.

c. Ligation of adapters to cDNA

After the *Eco*RI, *Sna*BI, *Xho*I adapters were ligated to the double-stranded cDNA using a standard protocol [see, *e.g.*, Sambrook *et al.* (1989) IN: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8], the

ligase was inactivated by heating the mixture to 72°C for 15 minutes. The following reagents were added to the cDNA ligation reaction and heated at 37°C for 30 minutes: cDNA ligation reaction (20 µl), water (24 µl), 10x kinase buffer (3 µl), 10 mM ATP (1 µl) and kinase (2µl of 2 U/µl). The reaction was stopped by the addition of 2 µl 0.5M EDTA, followed by one phenol/chloroform extraction and one chloroform extraction.

d. Size Selection and Packaging of cDNA

The double-stranded cDNA with the *Eco*RI, *Sna*BI, *Xho*I adapters ligated was purified away from the free or unligated adapters using a 5 ml Sepharose CL-4B column (Sigma, St. Louis, MO). 100 µl fractions were collected and those containing the cDNA, determined by monitoring the radioactivity, were pooled, ethanol precipitated, resuspended in TE buffer and loaded onto a 1% agarose gel. After the electrophoresis, the gel was stained with ethidium bromide and the 1 to 3 kb fraction was cut from the gel. The cDNA embedded in the agarose was eluted using the "Geneluter Electroelution System" (Invitrogen, San Diego, CA). The eluted cDNA was collected by ethanol precipitation and resuspended in TE buffer at 0.10 pmol/µl. The cDNA was ligated to 1 µg of *Eco*RI digested, dephosphorylated λgt11 in a 5 µl reaction volume at a 2- to 4- fold molar excess ratio of cDNA over the λgt11 vector. The ligated λgt11 containing the cDNA insert was packaged into λ phage virions *in vitro* using the Gigapack (Stratagene, La Jolla, CA) kit. The packaged phage were plated on an *E. coli* Y1088 bacterial lawn in preparation for screening.

2. IMR32 cDNA library #2

This library was prepared as described (Example I.B.1.) with the exception that 3 to 9 kb cDNA fragments were ligated into the λgt11 phage vector rather than the 1 to 3 kb fragments.

3. IMR32 cDNA library #3

IMR32 cell poly(A$^+$) RNA (Example I.A.1.) was used as a template to synthesize single-stranded cDNA. The primers for the first strand cDNA synthesis were random primers (hexadeoxy-nucleotides [pd(N)$_6$] Cat #5020-1, Clontech, Palo Alto, CA). The double-stranded cDNA was synthesized, *Eco*RI, *Sna*BI, *Xho*I adapters were added to the cDNA, the unligated adapters were removed, and the double-stranded cDNA with the ligated adapters was fractionated on an agarose gel, as described in Example I.B.1. The cDNA fraction greater than 1.8 kb was eluted from the agarose, ligated into λgt11, packaged, and plated into a bacterial lawn of Y1088 (as described in Example I.B.1.).

4. IMR32 cDNA library #4

IMR32 cell poly(A$^+$) RNA (Example I.A.1.) was used as a template to synthesize single-stranded cDNA. The primers for the first strand cDNA synthesis were oligonucleotides: 89-365a specific for the α_{1D} (VDCC III) type α_1-subunit (see Example II.A.) coding sequence (the complementary sequence of nt 2927 to 2956, SEQ ID No. 1), 89-495 specific for the α_{1C} (VDCC II) type α_1-subunit (see Example II.B.) coding sequence (the complementary sequence of nt 852 to 873, SEQ ID No. 3), and 90-12 specific for the α_{1C}-subunit coding sequence (the complementary sequence of nt 2496 to 2520, SEQ ID No. 3). The cDNA library was then constructed as described (Example I.B.3), except that the cDNA size-fraction greater than 1.5 kb was eluted from the agarose rather than the greater than 1.8 kb fraction.

5. IMR32 cDNA library #5

The cDNA library was constructed as described (Example I.B.3.) with the exception that the size-fraction greater than 1.2 kb was eluted from the agarose rather than the greater than 1.8 kb fraction.

6. Human thalamus cDNA library #6

Human thalamus poly (A$^+$) RNA (Example I.A.2.) was used as a template to synthesize single-stranded cDNA. Oligo (dT) was

used to prime the first strand synthesis (Example I.B.1.). The double-stranded cDNA was synthesized (Example I.B.1.) and *Eco*RI, *Kpn*I, *Nco*I adapters of the following sequence:
5′ CCATGGTACCTTCGTTGACG 3′= 20-mer (SEQ ID NO. 17)
3′ GGTACCATGGAAGCAACTGCTTAA 5′= 24-mer (SEQ ID NO. 18)
were ligated to the double-stranded cDNA as described (Example I.B.1.) with the 20-mer replacing the 18-mer and the 24-mer replacing the 22-mer. The unligated adapters were removed by passing the cDNA-adapter mixture through a 1 ml Bio Gel A-50 (Bio-Rad Laboratories, Richmond, CA) column. Fractions (30 µl) were collected and 1 µl of each fraction in the first peak of radioactivity was electrophoresed on a 1% agarose gel. After electrophoresis, the gel was dried on a vacuum gel drier and exposed to x-ray film. The fractions containing cDNA fragments greater than 600 bp were pooled, ethanol precipitated, and ligated into λgt11 (Example I.B.1.). The construction of the cDNA library was completed as described (Example I.B.1.).

C. Hybridization and Washing Conditions

Hybridization of radiolabelled nucleic acids to immobilized DNA for the purpose of screening cDNA libraries, DNA Southern transfers, or northern transfers was routinely performed in standard hybridization conditions [hybridization: 50% deionized formamide, 200 µg/ml sonicated herring sperm DNA (Cat #223646, Boehringer Mannheim Biochemicals, Indianapolis, IN), 5 x SSPE, 5 x Denhardt's, 42° C.; wash :0.2 x SSPE, 0.1% SDS, 65° C]. The recipes for SSPE and Denhardt's and the preparation of deionized formamide are described, for example, in Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8). In some hybridizations, lower stringency conditions were used in that 10% deionized formamide replaced 50% deionized formamide described for the standard hybridization conditions.

The washing conditions for removing the non-specific probe from the filters was either high, medium, or low stringency as described below:

1) high stringency: 0.1 x SSPE, 0.1% SDS, 65°C
2) medium stringency: 0.2 x SSPE, 0.1% SDS, 50°C
3) low stringency: 1.0 x SSPE, 0.1% SDS, 50°C.

It is understood that equivalent stringencies may be achieved using alternative buffers, salts and temperatures.

EXAMPLE II: ISOLATION OF DNA ENCODING THE HUMAN NEURONAL CALCIUM CHANNEL α_1 SUBUNIT

A. Isolation of DNA encoding the α_{1D} subunit

1. Reference list of partial α_{1D} cDNA clones

Numerous α_{1D}-specific cDNA clones were isolated in order to characterize the complete α_{1D} coding sequence plus portions of the 5′ and 3′ untranslated sequences. SEQ ID No. 1 shows the complete α_{1D} DNA coding sequence, plus 510 nucleotides of α_{1D} 5′ untranslated sequence ending in the guanidine nucleotide adjacent to the adenine nucleotide of the proposed initiation of translation as well as 642 nucleotides of 3′ untranslated sequence. Also shown in SEQ ID No. 1 is the deduced amino acid sequence. A list of partial cDNA clones used to characterize the α_{1D} sequence and the nucleotide position of each clone relative to the full-length α_{1D} cDNA sequence, which is set forth in SEQ ID No. 1, is shown below. The isolation and characterization of these clones are described below (Example II.A.2.).

IMR32	1.144	nt 1 to 510 of 5′ untranslated sequence,	SEQ ID No. 1
		nt 511 to 2431,	SEQ ID No. 1
IMR32*	1.136	nt 1627 to 2988,	SEQ ID No. 1
		nt 1 to 104 of additional exon,	SEQ ID No. 2
IMR32@	1.80	nt 2083 to 6468,	SEQ ID No. 1
IMR32#	1.36	nt 2857 to 4281,	SEQ ID No. 1
IMR32	1.163	nt 5200 to 7635,	SEQ ID No. 1

* 5' of nt 1627, IMR32 1.136 encodes an intron and an additional exon described in Example II.A.2.d.

@ IMR32 1.80 contains two deletions, nt 2984 to 3131 and nt 5303 to 5349 (SEQ ID No. 1). The 148 nt deletion (nt 2984 to 3131) was corrected by performing a polymerase chain reaction described in Example II.A.3.b.

IMR32 1.36 contains a 132 nt deletion (nt 3081 to 3212).

2. Isolation and characterization of individual clones listed in Example II.A.1.

a. IMR32 1.36

Two million recombinants of the IMR32 cDNA library #1 (Example I.B.1.) were screened in duplicate at a density of approximately 200,000 plaques per 150 mm plate using a mixture of radiolabelled fragments of the coding region of the rabbit skeletal muscle calcium channel α_1 cDNA [for the sequence of the rabbit skeletal muscle calcium channel α_1 subunit cDNA, see, Tanabe *et al.* (1987). *Nature* *328*:313-318]:

Fragment	**Nucleotides**
*Kpn*I-*Eco*RI	-78 to 1006
*Eco*RI-*Xho*I	1006 to 2653
*Apa*I-*Apa*I	3093 to 4182
*Bgl*II-*Sac*I	4487 to 5310

The hybridization was performed using low stringency hybridization conditions (Example I.C.) and the filters were washed under low stringency (Example I.C.). Only one α_{1D}-specific recombinant (IMR32 1.36) of the 2 x 10^6 screened was identified. IMR32 1.36 was plaque purified by standard methods (J. Sambrook *et al.* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Chapter 8) subcloned into pGEM3 (Promega, Madison, WI) and characterized by DNA sequencing.

b. IMR32 1.80

Approximately 1 x 10^6 recombinants of the IMR32 cDNA library #2 (Example I.B.2.) were screened in duplicate at a

density of approximately 100,000 plaques per 150 mm plate using the IMR32 1.36 cDNA fragment (Example II.A.1) as a probe. Standard hybridization conditions were used, and the filters were washed under high stringency (Example I.C.). Three positive plaques were identified one of which was IMR32 1.80. IMR32 1.80 was plaque purified by standard methods, restriction mapped, subcloned, and characterized by DNA sequencing.

c. IMR32 1.144

Approximately 1 x 10^6 recombinants of the IMR32 cDNA library #3 (Example I.B.3) were screened with the *Eco*RI-*Pvu*II fragment (nt 2083 to 2518, SEQ ID No. 1) of IMR32 1.80. The hybridization was performed using standard hybridization conditions (Example I.C.) and the filters were washed under high stringency (Example I.C.). Three positive plaques were identified one of which was IMR32 1.144. IMR32 1.144 was plaque purified, restriction mapped, and the cDNA insert was subcloned into pGEM7Z (Promega, Madison, WI) and characterized by DNA sequencing. This characterization revealed that IMR32 1.144 has a series of ATG codons encoding seven possible initiating methionines (nt 511 to 531, SEQ ID No. 1). Nucleic acid amplification analysis, and DNA sequencing of cloned nucleic acid amplification analysis products encoding these seven ATG codons confirmed that this sequence is present in the α_{1D} transcript expressed in dbcAMP-induced IMR32 cells.

d. IMR32 1.136

Approximately 1 x 10^6 recombinants of the IMR32 cDNA library #4 (Example I.B.4) were screened with the *Eco*RI-*Pvu*II fragment (nt 2083 to 2518, SEQ ID No. 1) of IMR32 1.80 (Example II.A.1.). The hybridization was performed using standard hybridization conditions (Example I.C.) and the filters were washed under high stringency (Example I.C.). Six positive plaques were identified one of which was IMR32 1.136. IMR32 1.136 was plaque purified, restriction mapped, and the cDNA insert was subcloned into a standard plasmid vector, pSP72 (Promega, Madison, WI.), and characterized by DNA

sequencing. This characterization revealed that IMR32 1.136 encodes an incompletely spliced α_{1D} transcript. The clone contains nucleotides 1627 to 2988 of SEQ ID No. 1 preceded by an approximate 640 bp intron. This intron is then preceded by a 104 nt exon (SEQ ID No. 2) which is an alternative exon encoding the IS6 transmembrane domain [see, *e.g.*, Tanabe *et al.* (1987) *Nature 328*:313-318 for a description of the IS1 to IVS6 transmembrane terminology] of the α_{1D} subunit and can replace nt 1627 to 1730, SEQ ID No. 1, to produce a completely spliced α_{1D} transcript.

e. IMR32 1.163

Approximately 1 x 10^6 recombinants of the IMR32 cDNA library #3 (Example I.B.3.) were screened with the *Nco*I-*Xho*I fragment of IMR32 1.80 (Example II.A.1.) containing nt 5811 to 6468 (SEQ ID No. 1). The hybridization was performed using standard hybridization conditions (Example I.C.) and the filters were washed under high stringency (Example I.C.). Three positive plaques were identified one of which was IMR32 1.163. IMR32 1.163 was plaque purified, restriction mapped, and the cDNA insert was subcloned into a standard plasmid vector, pSP72 (Promega, Madison, WI.), and characterized by DNA sequencing. This characterization revealed that IMR32 1.163 contains the α_{1D} termination codon, nt 6994 to 6996 (SEQ ID No. 1).

3. Construction of a full-length α_{1D} cDNA [pVDCCIII(A)]

α_{1D} cDNA clones IMR32 1.144, IMR32 1.136, IMR32 1.80, and IMR32 1.163 (Example II.A.2.) overlap and include the entire α_{1D} coding sequence, nt 511 to 6993 (SEQ ID No. 1), with the exception of a 148 bp deletion, nt 2984 to 3131 (SEQ ID No. 1). Portions of these partial cDNA clones were ligated to generate a full-length α_{1D} cDNA in a eukaryotic expression vector. The resulting vector was called pVDCCIII(A). The construction of pVDCCIII(A) was performed in four steps described in detail below: (1) the construction of pVDCCIII/5' using portions of IMR32 1.144, IMR32 1.136, and

IMR32 1.80, (2) the construction of pVDCCIII/5'.3 that corrects the 148 nt deletion in the IMR32 1.80 portion of pVDCCIII/5', (3) the construction of pVDCCIII/3'.1 using portions of IMR32 1.80 and IMR32 1.163, and (4) the ligation of a portion of the pVDCCIII/5'.3 insert, the insert of pVDCCIII/3'.1, and pcDNA1 (Invitrogen, San Diego, CA) to form pVDCCIII(A). The vector pcDNA1 is a eukaryotic expression vector containing a cytomegalovirus (CMV) promoter which is a constitutive promoter recognized by mammalian host cell RNA polymerase II.

Each of the DNA fragments used in preparing the full-length construct was purified by electrophoresis through an agarose gel onto DE81 filter paper (Whatman, Clifton, NJ) and elution from the filter paper using 1.0 M NaCl, 10 mM TRIS, pH 8.0, 1 mM EDTA. The ligations typically were performed in a 10 μl reaction volume with an equal molar ratio of insert fragment and a two-fold molar excess of the total insert relative to the vector. The amount of DNA used was normally about 50 ng to 100 ng.

a. pVDCCIII/5'

To construct pVDCCIII/5', IMR32 1.144 (Example II.A.2.c.) was digested with *Xho*I and *Eco*RI and the fragment containing the vector (pGEM7Z), α_{1D} nt 1 to 510 (SEQ ID No. 1), and α_{1D} nt 511 to 1732 (SEQ ID No. 1) was isolated by gel electrophoresis. The *Eco*RI-*Apa*I fragment of IMR32 1.136 (Example II.A.2.d.) nucleotides 1733 to 2671 (SEQ ID No. 1) was isolated, and the *Apa*I-*Hin*dIII fragment of IMR32 1.80 (Example II.A.2.b.), nucleotides 2672 to 4492 (SEQ ID No. 1) was isolated. The three DNA clones were ligated to form pVDCCIII/5' containing nt 1 to 510 (5' untranslated sequence; SEQ ID No. 1) and nt 511 to 4492 (SEQ ID No. 1).

b. pVDCCIII/5'.3

Comparison of the IMR32 1.36 and IMR32 1.80 DNA sequences revealed that these two cDNA clones differ through the α_{1D} coding sequence, nucleotides 2984 to 3212. nucleic acid amplification analysis of IMR32 1.80 and dbcAMP-induced

(1.0 mM, 10 days) IMR32 cytoplasmic RNA (isolated according to Ausubel, F.M. *et al.* (Eds) (1988) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York) revealed that IMR32 1.80 had a 148 nt deletion, nt 2984 to 3131 (SEQ ID No. 1), and that IMR32 1.36 had a 132 nt deletion, nt 3081 to 3212. To perform the nucleic acid amplification analysis, the amplification reaction was primed with α_{1D}-specific oligonucleotides 112 (nt 2548 to 2572, SEQ ID No. 1) and 311 (the complementary sequence of nt 3928 to 3957, SEQ ID No. 1). These products were then reamplified using α_{1D}-specific oligonucleotides 310 (nt 2583 to 2608 SEQ ID No. 1) and 312 (the complementary sequence of nt 3883 to 3909). This reamplified product, which contains *Acc*I and *Bgl*II restriction sites, was digested with *Acc*I and *Bgl*II and the *Acc*I-*Bgl*II fragment, nt 2765 to 3890 (SEQ ID No. 1) was cloned into *Acc*I-*Bgl*II digested pVDCCIII/5' to replace the *Acc*I-*Bgl*II pVDCCIII/5' fragment that had the deletion. This new construct was named pVDCCIII/5'.3. DNA sequence determination of pVDCCIII/5'.3 through the amplified region confirmed the 148 nt deletion in IMR32 1.80.

c. pVDCCIII/3'.1

To construct pVDCCIII/3'.1, the cDNA insert of IMR32 1.163 (Example II.A.2.e.) was subcloned into pBluescript II (Stratagene, La Jolla, CA) as an *Xho*I fragment. The *Xho*I sites on the cDNA fragment were furnished by the adapters used to construct the cDNA library (Example I.B.3.). The insert was oriented such that the translational orientation of the insert of IMR32 1.163 was opposite to that of the *lacZ* gene present in the plasmid, as confirmed by analysis of restriction enzyme digests of the resulting plasmid. This was done to preclude the possibility of expression of α_{1D} sequences in DH5α cells transformed with this plasmid due to fusion with the *lacZ* gene. This plasmid was then digested with *Hin*dIII and *Bgl*II and the *Hin*dIII - *Bgl*II fragment (the *Hin*dIII site comes from the vector and the *Bgl*II site is at nt 6220, SEQ ID No. 1) was eliminated, thus deleting nt 5200 to 6220 (SEQ ID

No. 1) of the IMR32 1.163 clone and removing this sequence from the remainder of the plasmid which contained the 3' *Bgl*II - *Xho*I fragment, nt 6221 to 7635 (SEQ ID No. 1). pVDCCIII/3'.1 was then made by splicing together the *Hin*dIII-*Pvu*II fragment from IMR32 1.80 (nucleotides 4493-5296, SEQ ID No. 1), the *Pvu*II - *Bgl*II fragment of IMR32 1.163 (nucleotides 5294 to 6220, SEQ ID No. 1) and the *Hin*dIII-*Bgl*II-digested pBluescript plasmid containing the 3' *Bgl*II/*Xho*I IMR32 1.163 fragment (nt 6221 to 7635, SEQ ID No. 1).

d. pVDCCIII(A): the full-length α_{1D} construct

To construct pVDCCIII(A), the *Dra*I-*Hin*dIII fragment (5' untranslated sequence nt 330 to 510, SEQ ID No. 1 and coding sequence nt 511 to 4492, SEQ ID No. 1) of pVDCCIII/5'.3 (Example II.A.3.b.) was isolated; the *Hin*dIII-*Xho*I fragment of pVDCCIII/3'.1 (containing nt 4493 to 7635, SEQ ID No. 1, plus the *Xho*I site of the adapter) (Example II.A.3.c.) was isolated; and the plasmid vector, pcDNA1, was digested with *Eco*RV and *Xho*I and isolated on an agarose gel. The three DNA fragments were ligated and MC1061-P3 (Invitrogen, San Diego, CA) was transformed. Isolated clones were analyzed by restriction mapping and DNA sequencing and pVDCCIII(A) was identified which had the fragments correctly ligated together: *Dra*I-*Hin*dIII, *Hin*dIII-*Xho*I, *Xho*I-*Eco*RV with the blunt-end *Dra*I and *Eco*RV site ligating together to form the circular plasmid.

The amino-terminus of the α_{1D} subunit is encoded by the seven consecutive 5' methionine codons (nt 511 to 531, SEQ ID No. 1). This 5' portion plus nt 532 to 537,
encoding two lysine residues, were deleted from pVDCCIII(A) and replaced with an efficient ribosomal binding site (5'-ACCACC-3') to form pVDCCIII.RBS(A). Expression experiments in which transcripts of this construct were injected into *Xenopus laevis* oöcytes did not result in an enhancement in the recombinant voltage-dependent calcium channel expression level relative to the level of expression in oöcytes injected with transcripts of pVDCCIII(A).

B. Isolation of DNA encoding the α_{1C} subunit

1. Reference List of Partial α_{1C} cDNA clones

Numerous α_{1C}-specific cDNA clones were isolated in order to characterize the α_{1C} coding sequence, the α_{1C} initiation of translation, and an alternatively spliced region of α_{1C}. SEQ ID No. 3 sets forth one α_{1C} coding sequence (α_{1C-1}) and deduced amino acid sequence; SEQ ID No. 36 sets forth another splice variant designated α_{1C-2}. SEQ ID No. 4 and No. 5 encode two possible amino terminal ends of an α_{1C} splice variant. SEQ ID No. 6 encodes an alternative exon for the IV S3 transmembrane domain. Other α_{1C} variants can be constructed by selecting the alternative amino terminal ends in place of the ends in SEQ ID No. 3 or 36 and/or inserting the alternative exon (SEQ ID No. 6) in the appropriate location, such as in SEQ ID NO. 3 in place of nucleotides 3904-3987. In addition, the 75 nucleotide sequence (nucleotides 1391-1465 in SEQ ID No. 3) can be deleted or inserted to produce an alternative α_{1C} splice variant.

Shown below is a list of clones used to characterize the α_{1C} sequence and the nucleotide position of each clone relative to the characterized α_{1C} sequence (SEQ ID No. 3). The isolation and characterization of these cDNA clones are described below (Example II.B.2).

IMR32	1.66	nt 1 to 916, SEQ ID No. 3 nt 1 to 132, SEQ ID No. 4
IMR32	1.157	nt 1 to 873, SEQ ID No. 3 nt 1 to 89, SEQ ID No. 5
IMR32	1.67	nt 50 to 1717, SEQ ID No. 3
*IMR32	1.86	nt 1366 to 2583, SEQ ID No. 3
©1.16G		nt 758 to 867, SEQ ID No. 3
IMR32	1.37	nt 2804 to 5904, SEQ ID No. 3
CNS	1.30	nt 2199 to 3903, SEQ ID No. 3 nt 1 to 84 of alternative exon, SEQ ID No. 6
IMR32	1.38	nt 2448 to 4702, SEQ ID No. 3 nt 1 to 84 of alternative exon,

SEQ ID No. 6

* IMR32 1.86 has a 73 nt deletion compared to the rabbit cardiac muscle calcium channel α_1 subunit cDNA sequence.

@1.16G is an α_{1C} genomic clone.

2. Isolation and characterization of clones described in Example II.B.1.

a. CNS 1.30

Approximately 1 x 10^6 recombinants of the human thalamus cDNA library No. 6 (Example I.B.6.) were screened with fragments of the rabbit skeletal muscle calcium channel α_1 cDNA described in Example II.A.2.a. The hybridization was performed using standard hybridization conditions (Example I.C.) and the filters were washed under low stringency (Example I.C.). Six positive plaques were identified, one of which was CNS 1.30. CNS 1.30 was plaque purified, restriction mapped, subcloned, and characterized by DNA sequencing. CNS 1.30 encodes α_{1C}-specific sequence nt 2199 to 3903 (SEQ ID No. 3) followed by nt 1 to 84 of one of two identified alternative α_{1C} exons (SEQ ID No. 6). 3′ of SEQ ID No. 6, CNS 1.30 contains an intron and, thus, CNS 1.30 encodes a partially spliced α_{1C} transcript.

b. 1.16G

Approximately 1 x 10^6 recombinants of a λEMBL3-based human genomic DNA library (Cat # HL1006d Clontech Corp., Palo Alto, CA) were screened using a rabbit skeletal muscle cDNA fragment (nt -78 to 1006, Example II.A.2.a.). The hybridization was performed using standard hybridization conditions (Example I.C.) and the filters were washed under low stringency (Example I.C.). Fourteen positive plaques were identified, one of which was 1.16G. Clone 1.16G was plaque purified, restriction mapped, subcloned, and portions were characterized by DNA sequencing. DNA sequencing revealed that 1.16G encodes α_{1C}-specific sequence as described in Example II.B.1.

c. IMR32 1.66 and IMR32 1.67

Approximately 1 x 10^6 recombinants of IMR32 cDNA library #5 (Example I.B.5.) were screened with a 151 bp *Kpn*I-*Sac*I fragment of 1.16G (Example II.B.2.b.) encoding α_{1C} sequence (nt 758 to 867, SEQ ID No. 3). The hybridization was performed using standard hybridization conditions (Example I.C.). The filters were then washed in 0.5 x SSPE at 65°C. Of the positive plaques, IMR32 1.66 and IMR32 1.67 were identified. The hybridizing plaques were purified, restriction mapped, subcloned, and characterized by DNA sequencing. Two of these cDNA clones, IMR32 1.66 and 1.67, encode α_{1C} subunits as described (Example II.B.1.). In addition, IMR32 1.66 encodes a partially spliced α_{1C} transcript marked by a GT splice donor dinucleotide beginning at the nucleotide 3' of nt 916 (SEQ ID No. 3). The intron sequence within 1.66 is 101 nt long. IMR32 1.66 encodes the α_{1C} initiation of translation, nt 1 to 3 (SEQ ID No. 3) and 132 nt of 5' untranslated sequence (SEQ ID No. 4) precede the start codon in IMR32 1.66.

d. IMR32 1.37 and IMR32 1.38

Approximately 2 x 10^6 recombinants of IMR32 cDNA library #1 (Example I.B.1.) were screened with the CNS 1.30 cDNA fragment (Example II.B.2.a.). The hybridization was performed using low stringency hybridization conditions (Example I.C.) and the filters were washed under low stringency (Example I.C.). Four positive plaques were identified, plaque purified, restriction mapped, subcloned, and characterized by DNA sequencing. Two of the clones, IMR32 1.37 and IMR32 1.38 encode α_{1C}-specific sequences as described in Example II.B.1.

DNA sequence comparison of IMR32 1.37 and IMR32 1.38 revealed that the α_{1C} transcript includes two exons that encode the IVS3 transmembrane domain. IMR32 1.37 has a single exon, nt 3904 to 3987 (SEQ ID No. 3) and IMR32 1.38 appears to be anomalously spliced to contain both exons juxtaposed, nt 3904 to 3987 (SEQ ID No. 3) followed by nt 1 to 84 (SEQ ID No. 6). The alternative splice of the α_{1C} transcript to contain either of the two exons encoding the IVS3 region was confirmed by

comparing the CNS 1.30 sequence to the IMR32 1.37 sequence. CNS 1.30 contains nt 1 to 84 (SEQ ID No. 6) preceded by the identical sequence contained in IMR32 1.37 for nt 2199 to 3903 (SEQ ID No. 3). As described in Example II.B.2.a., an intron follows nt 1 to 84 (SEQ ID No. 6). Two alternative exons have been spliced adjacent to nt 3903 (SEQ ID No. 3) represented by CNS 1.30 and IMR32 1.37.

e. IMR32 1.86

IMR32 cDNA library #1 (Example I.B.1.) was screened in duplicate using oligonucleotide probes 90-9 (nt 1462 to 1491, SEQ ID No. 3) and 90-12 (nt 2496 to 2520, SEQ ID No. 3). These oligonucleotide probes were chosen in order to isolate a clone that encodes the α_{1C} subunit between the 3' end of IMR32 1.67 (nt 1717, SEQ ID No. 3) and the 5' end of CNS 1.30 (nt 2199, SEQ ID No. 3). The hybridization conditions were standard hybridization conditions (Example I.C.) with the exception that the 50% deionized formamide was reduced to 20%. The filters were washed under low stringency (Example I.C.). Three positive plaques were identified one of which was IMR32 1.86. IMR32 1.86 was plaque purified, subcloned, and characterized by restriction mapping and DNA sequencing. IMR32 1.86 encodes α_{1C} sequences as described in Example II.B.1. Characterization by DNA sequencing revealed that IMR32 1.86 contains a 73 nt deletion compared to the DNA encoding rabbit cardiac muscle calcium channel α_1 subunit [Mikami *et al.* (1989) *Nature 340*:230], nt 2191 to 2263. These missing nucleotides correspond to nt 2176-2248 of SEQ ID No. 3. Because the 5'-end of CNS 1.30 overlaps the 3'-end of IMR32 1.86, some of these missing nucleotides, i.e., nt 2205-2248 of SEQ ID No. 3, are accounted for by CNS 1.30. The remaining missing nucleotides of the 73 nucleotide deletion in IMR32 1.86 (i.e., nt 2176-2204 SEQ ID No. 3) were determined by nucleic acid amplification analysis of dbcAMP-induced IMR32 cell RNA. The 73 nt deletion is a frame-shift mutation and, thus, needs to be corrected. The exact human sequence through this region, (which has been determined by the DNA sequence of

CNS 1.30 and nucleic acid amplification analysis of IMR32 cell RNA) can be inserted into IMR32 1.86 by standard methods, *e.g.*, replacement of a restriction fragment or site-directed mutagenesis.

f. IMR32 1.157

One million recombinants of IMR32 cDNA library #4 (Example I.B.4.) were screened with an *Xho*I-*Eco*RI fragment of IMR32 1.67 encoding α_{1C} nt 50 to 774 (SEQ ID No. 3). The hybridization was performed using standard hybridization conditions (Example I.C.). The filters were washed under high stringency (Example I.C.). One of the positive plaques identified was IMR32 1.157. This plaque was purified, the insert was restriction mapped and subcloned to a standard plasmid vector pGEM7Z (Promega, Madison, WI). The DNA was characterized by sequencing. IMR32 1.157 appears to encodes an alternative 5′ portion of the α_{1C} sequence beginning with nt 1 to 89 (SEQ ID No. 5) and followed by nt 1 to 873 (SEQ ID No. 3). Analysis of the 1.66 and 1.157 5′ sequence is described below (Example II.B.3.).

3. Characterization of the α_{1C} initiation of translation site

Portions of the sequences of IMR32 1.157 (nt 57 to 89, SEQ ID No. 5; nt 1 to 67, SEQ ID No. 3), IMR32 1.66 (nt 100 to 132, SEQ ID No. 4; nt 1 to 67, SEQ ID No. 3), were compared to the rabbit lung CaCB-receptor cDNA sequence, nt -33 to 67 [Biel *et al.* (1990) *FEBS Lett. 269*:409]. The human sequences are possible alternative 5′ ends of the α_{1C} transcript encoding the region of initiation of translation. IMR32 1.66 closely matches the CaCB receptor cDNA sequence and diverges from the CaCB receptor cDNA sequence in the 5′ direction beginning at nt 122 (SEQ ID No. 4). The start codon identified in the CaCB receptor cDNA sequence is the same start codon used to describe the α_{1C} coding sequence, nt 1 to 3 (SEQ ID No. 3).

The sequences of α_{1C} splice variants, designated α_{1C-1} and α_{1C-2} are set forth in SEQ ID NOs. 3 and 36.

C. Isolation of partial cDNA clones encoding the α_{1B} subunit and construction of a full-length clone

A human basal ganglia cDNA library was screened with the rabbit skeletal muscle α_1 subunit cDNA fragments (see Example II.A.2.a for description of fragments) under low stringency conditions. One of the hybridizing clones was used to screen an IMR32 cell cDNA library to obtain additional partial α_{1B} cDNA clones, which were in turn used to further screen an IMR32 cell cDNA library for additional partial cDNA clones. One of the partial IMR32 α_{1B} clones was used to screen a human hippocampus library to obtain a partial α_{1B} clone encoding the 3′ end of the α_{1B} coding sequence. The sequence of some of the regions of the partial cDNA clones was compared to the sequence of products of nucleic acid amplification analysis of IMR32 cell RNA to determine the accuracy of the cDNA sequences.

Nucleic acid amplification analysis analysis of IMR32 cell RNA and genomic DNA using oligonucleotide primers corresponding to sequences located 5′ and 3′ of the STOP codon of the DNA encoding the α_{1B} subunit revealed an alternatively spliced α_{1B}-encoding mRNA in IMR32 cells. This second mRNA product is the result of differential splicing of the α_{1B} subunit transcript to include another exon that is not present in the mRNA corresponding to the other 3′ α_{1B} cDNA sequence that was initially isolated. To distinguish these splice variants of the α_{1B} subunit, the subunit encoded by a DNA sequence corresponding to the form containing the additional exon is referred to as α_{1B-1} (SEQ ID No. 7), whereas the subunit encoded by a DNA sequence corresponding to the form lacking the additional exon is referred to as α_{1B-2} (SEQ ID No. 8). The sequence of α_{1B-1} diverges from that of α_{1B-2} beginning at nt 6633 (SEQ ID No. 7). Following the sequence of the additional exon in α_{1B-1} (nt 6633-6819; SEQ ID No. 7), the α_{1B-1} and α_{1B-2} sequences are identical (i.e., nt 6820-7362 in SEQ ID No. 7 and nt 6633-7175 in SEQ ID No. 8). SEQ ID No. 7 and No. 8 set forth 143 nt of 5′ untranslated sequence (nt 1-143) as well as

202 nt of 3′ untranslated sequence (nt 7161-7362, SEQ ID No. 7) of the DNA encoding α_{1B-1} and 321 nt of 3′ untranslated sequence (nt 6855-7175, SEQ ID No. 8) of the DNA encoding α_{1B-2}.

Nucleic acid amplification analysis analysis of the IS6 region of the α_{1B} transcript revealed what appear to be additional splice variants based on multiple fragment sizes seen on an ethidium bromide-stained agarose gel containing the products of the amplification reaction.

A full-length α_{1B-1} cDNA clone designated pcDNA-α_{1B-1} was prepared in an eight-step process as follows.

STEP 1: The *Sac*I restriction site of pGEM3 (Promega, Madison, WI) was destroyed by digestion at the *Sac*I site, producing blunt ends by treatment with T4 DNA polymerase, and religation. The new vector was designated pGEMΔSac.

STEP 2: Fragment 1 (*Hind*III/*Kpn*I; nt 2337 to 4303 of SEQ ID No. 7) was ligated into *Hind*III/*Kpn*I digested pGEM3ΔSac to produce pα1.177HK.

STEP 3: Fragment 1 has a 2 nucleotide deletion (nt 3852 and 3853 of SEQ ID No. 7). The deletion was repaired by inserting an amplfied fragment (fragment 2) of IMR32 RNA into pα1.177HK. Thus, fragment 2 (*Nar*I/*Kpn*I; nt 3828 to 4303 of SEQ ID No. 7) was inserted into *Nar*I/*Kpn*I digested pα1.177HK replacing the *Nar*I/*Kpn*I portion of fragment 1 and producing pα1.177HK/PCR.

STEP 4: Fragment 3 (*Kpn*I/*Kpn*I; nt 4303 to 5663 of SEQ ID No. 7) was ligated into *Kpn*I digested pα1.177HK/PCR to produce pα1B5′K.

STEP 5: Fragment 4 (*Eco*RI/*Hin*dIII; *Eco*RI adaptor plus nt 1 to 2337 of SEQ ID No. 7) and fragment 5 (*Hin*dIII/*Xho*I fragment of pα1B5′K; nt 2337 to 5446 of SEQ ID No. 7) were ligated together into *Eco*RI/*Xho*I digested pcDNA1 (Invitrogen, San Diego, CA) to produce pα1B5′.

STEP 6: Fragment 6 (*Eco*RI/*Eco*RI; *Eco*RI adapters on both ends plus nt 5749 to 7362 of SEQ ID No. 7) was ligated into *Eco*RI digested pBluescript II KS (Stratagene, La Jolla, CA) with the 5′ end of the fragment proximal to the *Kpn*I site in the polylinker to produce pα1.230.

STEP 7: Fragment 7 (*Kpn*I/*Xho*I; nt 4303 to 5446 of SEQ ID No. 7), and fragment 8 (*Xho*I/*Csp*I; nt 5446 to 6259 of SEQ ID No. 7) were ligated into *Kpn*I/*Csp*I digested pα1.230 (removes nt 5749 to 6259 of SEQ ID No. 7 that was encoded in pα1.230 and maintains nt 6259 to 7362 of SEQ ID No. 7) to produce pα1B3′.

STEP 8: Fragment 9 (*Sph*I/*Xho*I; nt 4993 to 5446 of SEQ ID No. 7) and fragment 10 (*Xho*I/*Xba*I of pα1B3′; nt 5446 to 7319 of SEQ ID No. 7) were ligated into *Sph*I/*Xba*I digested pα1B5′ (removes nt 4993 to 5446 of SEQ ID No. 7 that were encoded in pα1B5′ and maintains nt 1 to 4850 of SEQ ID No. 7) to produce pcDNAα_{1B-1}.

The resulting construct, pcDNAα_{1B-1}, contains, in pCDNA1, a full-length coding region encoding α_{1B-1} (nt 144-7362, SEQ ID No. 7), plus 5′ untranslated sequence (nt 1-143, SEQ ID No. 7) and 3′ untranslated sequence (nt 7161-7319, SEQ ID No. 7) under the transcriptional control of the CMV promoter.

D. Isolation of DNA encoding human calcium channel α_{1A} subunits

1. Isolation of partial clones

DNA clones encoding portions of human calcium channel α_{1A} subunits were obtained by hybridization screening of human cerebellum cDNA libraries and nucleic acid amplification of human cerebellum RNA. Clones corresponding to the 3′ end of the α_{1A} coding sequence were isolated by screening 1 x 10^6 recombinants of a randomly primed cerebellum cDNA library (size-selected for inserts greater than 2.8 kb in length) under low stringency conditions (6X SSPE, 5X Denhart's solution, 0.2% SDS, 200 μg/ml sonicated herring sperm DNA,

42°C) with oligonucleotide 704 containing nt 6190-6217 of the rat α_{1A} coding sequence [Starr *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A. 88*:5621-5625]. Washes were performed under low stringency conditions. Several clones that hybridized to the probe (clones α1.251-α1.259 and α1.244) were purified and characterized by restriction enzyme mapping and DNA sequence analysis. At least two of the clones, α1.244 and α1.254, contained a translation termination codon. Although clones α1.244 and α1.254 are different lengths, they both contain a sequence of nucleotides that corresponds to the extreme 3' end of the α_{1A} transcript, i.e., the two clones overlap. These two clones are identical in the region of overlap, except, clone α1.244 contains a sequence of 5 and a sequence of 12 nucleotides that are not present in α1.254.

To obtain additional α_{1A}-encoding clones, 1 x 10^6 recombinants of a randomly primed cerebellum cDNA library (size-selected for inserts ranging from 1.0 to 2.8 kb in length) was screened for hybridization to three oligonucleotides: oligonucleotide 701 (containing nucleotides 2288-2315 of the rat α_{1A} coding sequence), oligonucleotide 702 (containing nucleotides 3559-3585 of the rat α_{1A} coding sequence) and oligonucleotide 703 (containing nucleotides 4798-4827 of the rat α_{1A} coding sequence). Hybridization and washes were performed using the same conditions as used for the first screening with oligonucleotide 704, except that washes were conducted at 45°C. Twenty clones (clones α1.269-α1.288) hybridized to the probe. Several clones were plaque-purified and characterized by restriction enzyme mapping and DNA sequence analysis. One clone, α1.279, contained a sequence of about 170 nucleotides that is not present in other clones corresponding to the same region of the coding sequence. This region may be present in other splice variants. None of the clones contained a translation intiation codon.

To obtain clones corresponding to the 5' end of the human α_{1A} coding sequence, another cerebellum cDNA library was

prepared using oligonucleotide 720 (containing nucleotides 2485-2510 of SEQ ID No. 22) to specifically prime first-strand cDNA synthesis. The library (8 x 10^5 recombinants) was screened for hybridization to three oligonucleotides: oligonucleotide 701, oligonucleotide 726 (containing nucleotides 2333-2360 of the rat α_{1A} coding sequence) and oligonucleotide 700 (containing nucleotides 767-796 of the rat α_{1A} coding sequence) under low stringency hybridization and washing conditions. Approximately 50 plaques hybridized to the probe. Hybridizing clones α1.381-α1.390 were plaque-purified and characterized by restriction enzyme maping and DNA sequence analysis. At least one of the clones, α1.381, contained a translation initiation codon.

Alignment of the sequences of the purified clones revealed that the sequences overlapped to comprise the entire α_{1A} coding sequence. However, not all the overlapping sequences of partial clones contained convenient enzyme restriction sites for use in ligating partial clones to construct a full-length α_{1A} coding sequence. To obtain DNA fragments containing convenient restriction enzyme sites that could be used in constructing a full-length α_{1A} DNA, cDNA was synthesized from RNA isolated from human cerebellum tissue and subjected to nucleic acid amplification. The oligonucleotides used as primers corresponded to human α_{1A} coding sequence located 5′ and 3′ of selected restriction enzyme sites. Thus, in the first amplification reaction, oligonucleotides 753 (containing nucleotides 2368-2391 of SEQ ID No. 22) and 728 (containing nucleotides 3179-3202 of SEQ ID No. 22) were used as the primer pair. To provide a sufficient amount of the desired DNA fragment, the product of this amplification was reamplified using oligonucleotides 753 and 754 (containing nucleotides 3112-3135 of SEQ ID No. 22 as the primer pair. The resulting product was 768 bp in length. In the second amplification reaction, oligonucleotides 719 (containing nucleotides 4950-4975 of SEQ ID No. 22 and 752 (containing nucleotides 5647-5670 of SEQ ID No. 22) were used as the

primer pair. To provide a sufficient amount of the desired second DNA fragment, the product of this amplification was reamplified using oligonucleotides 756 (containing nucleotides 5112-5135 of SEQ ID No. 22) and 752 as the primer pair. The resulting product was 559 bp in length.

2. Construction of full-Length α_{1A} coding sequences

Portions of clone α1.381, the 768-bp nucleic acid amplification product, clone α1.278, the 559-bp nucleic acid amplification product, and clone α1.244 were ligated at convenient restriction sites to generate a full-length α_{1A} coding sequence referred to as α_{1A-1}.

Comparison of the results of sequence analysis of clones α1.244 and α1.254 indicated that the primary transcript of the α_{1A} subunit gene is alternatively spliced to yield at least two variant mRNAs encoding different forms of the α_{1A} subunit. One form, α_{1A-1}, is encoded by the sequence shown in SEQ ID No. 22. The sequence encoding a second form, α_{1A-2}, differs from the α_{1A-1}-encoding sequence at the 3′ end in that it lacks a 5-nt sequence found in clone α1.244 (nucleotides 7035-7039 of SEQ ID No. 22). This deletion shifts the reading frame and introduces a translation termination codon resulting in an α_{1A-2} coding sequence that encodes a shorter α_{1A} subunit than that encoded by the α_{1A-1} splice variant. Consequently, a portion of the 3′ end of the α_{1A-1} coding sequence is actually 3′ untranslated sequence in the α_{1A-2} DNA. The complete sequence of α_{1A-2}, which can be constructed by ligating portions of clone α1.381, the 768-bp nucleic acid amplification product, clone α1.278, the 559-bp nucleic acid amplification product and clone α1.254, is set forth in SEQ ID No. 23.

E. Isolation of DNA Encoding the α_{1E} Subunit

DNA encoding α_{1E} subunits of the human calcium channel were isolated from human hippocampus libraries. The selected clones sequenced. DNA sequence analysis of DNA clones encoding the α_{1E} subunit indicated that at least two alternatively spliced forms of the same α_{1E} subunit primary transcript are expressed. One form has the sequence set forth

in SEQ ID No. 24 and was designated α_{1E-1} and the other was designated α_{1E-3}, which has the sequence obtained by inserting a 57 base pair fragment between nucleotides 2405 and 2406 of SEQ ID No. 24. The resulting sequence is set forth in SEQ ID No. 25.

The subunit designated α_{1E-1} has a calculated molecular weight of 254,836 and the subunit designated α_{1E-3} has a calculated molecular weight of 257,348. α_{1E-3} has a 19 amino acid insertion (encoded by SEQ ID No. 25) relative to α_{1E-1} in the region that appears to be the cytoplasmic loop between transmembrane domains IIS6 and IIIS1.

EXAMPLE III: ISOLATION OF cDNA CLONES ENCODING THE HUMAN NEURONAL CALCIUM CHANNEL β_1 subunit

A. Isolation of partial cDNA clones encoding the β subunit and construction of a full-length clone encoding the β_1 subunit

A human hippocampus cDNA library was screened with the rabbit skeletal muscle calcium channel β_1 subunit cDNA fragment (nt 441 to 1379) [for isolation and sequence of the rabbit skeletal muscle calcium channel β_1 subunit cDNA, see U.S. Patent Application Serial NO. 482,384 or Ruth *et al.* (1989) *Science 245*:1115] using standard hybridization conditions (Example I.C.). A portion of one of the hybridizing clones was used to rescreen the hippocampus library to obtain additional cDNA clones. The cDNA inserts of hybridizing clones were characterized by restriction mapping and DNA sequencing and compared to the rabbit skeletal muscle calcium channel β_1 subunit cDNA sequence.

Portions of the partial β_1 subunit cDNA clones were ligated to generate a full-length clone encoding the entire β_1 subunit. SEQ ID No. 9 shows the β_1 subunit coding sequence (nt 1-1434) as well as a portion of the 3' untranslated sequence (nt 1435-1546). The deduced amino acid sequence is also provided in SEQ ID No. 9. In order to perform expression experiments, full-length β_1 subunit cDNA clones were constructed as follows.

Step 1: DNA fragment 1 (~800 bp of 5′ untranslated sequence plus nt 1-277 of SEQ ID No. 9) was ligated to DNA fragment 2 (nt 277-1546 of SEQ ID No. 9 plus 448 bp of intron sequence) and cloned into pGEM7Z. The resulting plasmid, pβ1-1.18, contained a full-length β_1 subunit clone that included a 448-bp intron.

Step 2: To replace the 5′ untranslated sequence of pβ1-1.18 with a ribosome binding site, a double-stranded adapter was synthesized that contains an *Eco*RI site, sequence encoding a ribosome binding site (5′-ACCACC-3′) and nt 1-25 of SEQ ID No. 9. The adapter was ligated to *Sma*I-digested pβ1-1.18, and the products of the ligation reaction were digested with *Eco*RI.

Step 3: The *Eco*RI fragment from step 2 containing the *Eco*RI adapter, efficient ribosome binding site and nt 1-1546 of SEQ ID No. 9 plus intron sequence was cloned into a plasmid vector and designated pβ1-1.18RBS. The *Eco*RI fragment of pβ1-1.18RBS was subcloned into *Eco*RI-digested pcDNA1 with the initiation codon proximal to CMV promoter to form pHBCaCHβ_{1a}RBS(A).

Step 4: To generate a full-length clone encoding the β_1 subunit lacking intron sequence, DNA fragment 3 (nt 69-1146 of SEQ ID No. 9 plus 448 bp of intron sequence followed by nt 1147-1546 of SEQ ID No. 9), was subjected to site-directed mutagenesis to delete the intron sequence, thereby yielding pβ1(-). The *Eco*RI-*Xho*I fragment of pβ1-1.18RBS (containing of the ribosome binding site and nt 1-277 of SEQ ID No. 9) was ligated to the *Xho*I-*Eco*RI fragment of pβ1(-) (containing of nt 277-1546 of SEQ ID No. 9) and cloned into pcDNA1 with the initiation of translation proximal to the CMV promoter. The resulting expression plasmid was designated pHBCaCHβ_{1b}RBS(A).

B. Splice Variant β_{1-3}

DNA sequence analysis of the DNA clones encoding the β_1 subunit indicated that in the CNS at least two alternatively spliced forms of the same human β_1 subunit primary transcript are expressed. One form is represented by the sequence shown

in SEQ ID No. 9 and is referred to as β_{1-2}. The sequences of β_{1-2} and the alternative form, β_{1-3}, diverge at nt 1334 (SEQ ID No. 9). The complete β_{1-3} sequence (nt 1-1851), including 3' untranslated sequence (nt 1795-1851), is set forth in SEQ ID No. 10.

EXAMPLE IV: ISOLATION OF cDNA CLONES ENCODING THE HUMAN NEURONAL CALCIUM CHANNEL α_2-subunit

A. Isolation of cDNA clones

The complete human neuronal α_2 coding sequence (nt 35-3310) plus a portion of the 5' untranslated sequence (nt 1 to 34) as well as a portion of the 3' untranslated sequence (nt 3311-3600) is set forth in SEQ ID No. 11.

To isolate DNA encoding the human neuronal α_2 subunit, human α_2 genomic clones first were isolated by probing human genomic Southern blots using a rabbit skeletal muscle calcium channel α_2 subunit cDNA fragment [nt 43 to 272, Ellis *et al.* (1988) *Science 240*:1661]. Human genomic DNA was digested with *Eco*RI, electrophoresed, blotted, and probed with the rabbit skeletal muscle probe using standard hybridization conditions (Example I.C.) and low stringency washing conditions (Example I.C.). Two restriction fragments were identified, 3.5 kb and 3.0 kb. These *Eco*RI restriction fragments were cloned by preparing a λgt11 library containing human genomic *Eco*RI fragments ranging from 2.2 kb to 4.3 kb. The library was screened as described above using the rabbit α_2 probe, hybridizing clones were isolated and characterized by DNA sequencing. HGCaCHα2.20 contained the 3.5 kb fragment and HGCaCHα2.9 contained the 3.0 kb fragment.

Restriction mapping and DNA sequencing revealed that HGCaCHα2.20 contains an 82 bp exon (nt 130 to 211 of the human α_2 coding sequence, SEQ ID No. 11) on a 650 bp *Pst*I-*Xba*I restriction fragment and that HGCaCHα2.9 contains 105 bp of an exon (nt 212 to 316 of the coding sequence, SEQ ID No. 11) on a 750 bp *Xba*I-*Bgl*II restriction fragment. These restriction fragments were used to screen the human basal ganglia cDNA library (Example II.C.2.a.). HBCaCHα2.1 was isolated (nt 29

to 1163, SEQ ID No. 11) and used to screen a human brain stem cDNA library (ATCC Accession No. 37432) obtained from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD. 20852. Two clones were isolated, HBCaCHα2.5 (nt 1 to 1162, SEQ ID No. 11) and HBCaCHα2.8 (nt 714 to 1562, SEQ ID No. 11, followed by 1600 nt of intervening sequence). A 2400 bp fragment of HBCaCHα2.8 (beginning at nt 759 of SEQ ID No. 11 and ending at a *Sma*I site in the intron) was used to rescreen the brain stem library and to isolate HBCaCHα2.11 (nt 879 to 3600, SEQ ID No. 11). Clones HBCaCHα2.5 and HBCaCHα2.11 overlap to encode an entire human brain α_2 protein.

B. Construction of pHBCaCHα_2A

To construct pHBCaCHα_2A containing DNA encoding a full-length human calcium channel α_2 subunit, an (*Eco*RI)-*Pvu*II fragment of HBCaCHα2.5 (nt 1 to 1061, SEQ ID No. 11, *Eco*RI adapter, *Pvu*II partial digest) and a *Pvu*II-*Pst*I fragment of HBCaCHα2.11 (nt 1061 to 2424 SEQ ID No. 11; *Pvu*II partial digest) were ligated into *Eco*RI-*Pst*I-digested pIBI24 (Stratagene, La Jolla, CA). Subsequently, an (*Eco*RI)-*Pst*I fragment (nt 1 to 2424 SEQ ID No. 11) was isolated and ligated to a *Pst*I-(*Eco*RI) fragment (nt 2424 to 3600 SEQ ID No. 11) of HBCaCHα2.11 in *Eco*RI-digested pIBI24 to produce DNA, HBCaCHα2, encoding a full-length human brain α_2 subunit. The 3600 bp *Eco*RI insert of HBCaCHα2 (nt 1 to 3600, SEQ ID No. 11) was subcloned into pcDNA1 (pHBCaCHα2A) with the methionine initiating codon proximal to the CMV promoter. The 3600 bp *Eco*RI insert of HBCaCHα2 was also subcloned into pSV2dHFR [Subramani *et al.* (1981). *Mol. Cell. Biol.* 1:854-864] which contains the SV40 early promoter, mouse dihydrofolate reductase (dhfr) gene, SV40 polyadenylation and splice sites and sequences required for maintenance of the vector in bacteria.

EXAMPLE V. DIFFERENTIAL PROCESSING OF THE HUMAN β_1 TRANSCRIPT AND THE HUMAN α_2 TRANSCRIPT

A. Differential processing of the β_1 transcript

Nucleic acid amplification analysis of the human β_1 transcript present in skeletal muscle, aorta, hippocampus and basal ganglia, and HEK 293 cells revealed differential processing of the region corresponding to nt 615-781 of SEQ ID No. 9 in each of the tissues. Four different sequences that result in five different processed β_1 transcripts through this region were identified. The β_1 transcripts from the different tissues contained different combinations of the four sequences, except for one of the β_1 transcripts expressed in HEK 293 cells (β_{1-5}) which lacked all four sequences.

None of the β_1 transcripts contained each of the four sequences; however, for ease of reference, all four sequences are set forth end-to-end as a single long sequence in SEQ ID No. 12. The four sequences that are differentially processed are sequence 1 (nt 14-34 in SEQ ID No. 12), sequence 2 (nt 35-55 in SEQ ID No. 12), sequence 3 (nt 56-190 in SEQ ID No. 12) and sequence 4 (nt 191-271 in SEQ ID No. 12). The forms of the β_1 transcript that have been identified include: (1) a form that lacks sequence 1 called β_{1-1} (expressed in skeletal muscle), (2) a form that lacks sequences 2 and 3 called β_{1-2} (expressed in CNS), (3) a form that lacks sequences 1, 2 and 3 called β_{1-4} (expressed in aorta and HEK cells) and (4) a form that lacks sequences 1-4 called β_{1-5} (expressed in HEK cells). Additionally, the β_{1-4} and β_{1-5} contain a guanine nucleotide (nt 13 in SEQ ID No. 12) that is absent in the β_{1-1} and β_{1-2} forms. The sequences of β_1 splice variants are set forth in SEQ ID Nos. 9, 10 and 33-35.

B. Differential processing of transcripts encoding the α_2 subunit.

The complete human neuronal α_2 coding sequence (nt 35-3307) plus a portion of the 5′ untranslated sequence (nt 1 to 34) as well as a portion of the 3′ untranslated sequence (nt 3308-3600) is set forth as SEQ ID No. 11.

Nucleic acid amplification analysis of the human α_2 transcript present in skeletal muscle, aorta, and CNS revealed differential processing of the region corresponding to nt 1595-1942 of SEQ ID No. 11 in each of the tissues.

The analysis indicated that the primary transcript of the genomic DNA that includes the nucleotides corresponding to nt 1595-1942 also includes an additional sequence (SEQ ID No. 13: 5'CCTATTGGTGTAGGTATACCAACAATTAATTT AAGAAAAAGGAGACCCAATATCCAG 3') inserted between nt 1624 and 1625 of SEQ ID No. 11. Five alternatively spliced variant transcripts that differ in the presence or absence of one to three different portions of the region of the primary transcript that includes the region of nt 1595-1942 of SEQ ID No. 11 plus SEQ ID No. 13 inserted between nt 1624 and 1625 have been identified. The five α_2-encoding transcripts from the different tissues include different combinations of the three sequences, except for one of the α_2 transcripts expressed in aorta which lacks all three sequences. None of the α_2 transcripts contained each of the three sequences. The sequences of the three regions that are differentially processed are sequence 1 (SEQ ID No. 13), sequence 2 (5' AACCCCAAATCTCAG 3', which is nt 1625-1639 of SEQ ID No. 11), and sequence 3 (5' CAAAAAAGGGCAAAATGAAGG 3', which is nt 1908-1928 of SEQ ID No. 11). The five α_2 forms identified are (1) a form that lacks sequence 3 called α_{2a} (expressed in skeletal muscle), (2) a form that lacks sequence 1 called α_{2b} (expressed in CNS), (3) a form that lacks sequences 1 and 2 called α_{2c} expressed in aorta), (4) a form that lacks sequences 1, 2 and 3 called α_{2d} (expressed in aorta) and (5) a form that lacks sequences 1 and 3 called α_{2e} (expressed in aorta).

The sequences of α_{2a} - α_{2e} are set forth in SEQ. ID Nos. 29 - 32, respectively.

EXAMPLE VI: ISOLATION OF DNA ENCODING A CALCIUM CHANNEL γ SUBUNIT FROM A HUMAN BRAIN cDNA LIBRARY

A. Isolation of DNA encoding the γ subunit

Approximately 1 x 10^6 recombinants from a λgt11-based human hippocampus cDNA library (Clontech catalog #HL1088b, Palo Alto, CA) were screened by hybridization to a 484 bp sequence of the rabbit skeletal muscle calcium channel γ subunit cDNA (nucleotides 621-626 of the coding sequence plus 438 nucleotides of 3'-untranslated sequence) contained in vector γJ10 [Jay, S. *et al.* (1990). *Science 248*:490-492]. Hybridization was performed using moderate stringency conditions (20% deionized formamide, 5x Denhardt's, 6 x SSPE, 0.2% SDS, 20 μg/ml herring sperm DNA, 42°C) and the filters were washed under low stringency (see Example I.C.). A plaque that hybridized to this probe was purified and insert DNA was subcloned into pGEM7Z. This cDNA insert was designated γ1.4.

B. Characterization of γ1.4

γ1.4 was confirmed by DNA hybridization and characterized by DNA sequencing. The 1500 bp *Sst*I fragment of γ1.4 hybridized to the rabbit skeletal muscle calcium channel γ subunit cDNA γJ10 on a Southern blot. SEQ analysis of this fragment revealed that it contains of approximately 500 nt of human DNA sequence and ~1000 nt of λgt11 sequence (included due to apparent destruction of one of the *Eco*RI cloning sites in λgt11). The human DNA sequence contains of 129 nt of coding sequence followed immediately by a translational STOP codon and 3' untranslated sequence (SEQ ID No. 14).

To isolate the remaining 5' sequence of the human γ subunit cDNA, human CNS cDNA libraries and/or preparations of mRNA from human CNS tissues can first be assayed by nucleic acid amplification analysis methods using oligonucleotide primers based on the γ cDNA-specific sequence of γ1.4. Additional human neuronal γ subunit-encoding DNA can be isolated from cDNA libraries that, based on the results of the nucleic acid amplification analysis assay, contain γ-specific

amplifiable cDNA. Alternatively, cDNA libraries can be constructed from mRNA preparations that, based on the results of the nucleic acid amplification analysis assays, contain γ-specific amplifiable transcripts. Such libraries are constructed by standard methods using oligo dT to prime first-strand cDNA synthesis from poly A^+ RNA (see Example I.B.). Alternatively, first-strand cDNA can be specified by priming first-strand cDNA synthesis with a γ cDNA-specific oligonucleotide based on the human DNA sequence in γ1.4. A cDNA library can then be constructed based on this first-strand synthesis and screened with the γ-specific portion of γ1.4.

EXAMPLE VII: ISOLATION OF cDNA CLONES ENCODING THE HUMAN NEURONAL Ca CHANNEL β_2 SUBUNIT

Isolation of DNA Encoding human calcium channel β_2 subunits

Sequencing of clones isolated as described in Example III revealed a clone encoding a human neuronal calcium channel β_2 subunit (designated β_{2D} see, SEQ ID No. 26). An oligonucleotide based on the 5′ end of this clone was used to prime a human hippocampus cDNA library. The library was screened with this β_2 clone under conditions of low to medium stringency (final wash 0.5 X SSPE, 50° C). Several hybridizing clones were isolated and sequenced. Among these clones were those that encode β_{2C}, β_{2D} and β_{2E}. For example, the sequence of β_{2C} is set forth in SEQ ID NO. 37, and the sequeence of β_{2E} is set forth in SEQ ID No. 38.

A randomly primed hippocampus library was then screened using a combination of the clone encoding β_{2D} and a portion of the β_3 clone deposited under ATCC Accession No. 69048. Multiple hybridizing clones were isolated. Among these were clones designated β101, β102 and β104. β101 appears to encodes the 5′ end of a splice variant of β_2, designated β_{2E}. β102 and β104 encode portions of the 3′ end of β_2.

It appears that the β_2 splice variants include nucleotides 182-2294 of SEQ ID No. 26 and differ only between

the start codon and nucleotides that correspond to 212 of SEQ. ID No. 26.

EXAMPLE VIII: ISOLATION OF cDNA CLONES ENCODING HUMAN CALCIUM CHANNEL β_4 and β_3 SUBUNITS

A. Isolation of cDNA Clones Encoding a Human β_4 Subunit

A clone containing a translation initiation codon and approximately 60% of the β_4 coding sequence was obtained from a human cerebellum cDNA library (see nucleotides 1-894 of Sequence ID No. 27). To obtain DNA encoding the remaining 3' portion of the β_4 coding sequence, a human cerebellum cDNA library was screened for hybridization a nucleic acid amplification product under high stringency hybridization and wash conditions. Hybridizing clones are purified and characterized by restriction enzyme mapping and DNA sequence analysis to identify those that contain sequence corresponding to the 3' end of the β_4 subunit coding sequence and a termination codon. Selected clones are ligated to the clone containing the 5' half of the β_4 coding sequence at convenient restriction sites to generate a full-length cDNA encoding a β_4 subunit. The sequence of a full-length β_4 clone is set forth in SEQ ID No. 27; the amino acid sequence is set forth in SEQ ID No. 28.

B. Isolation of cDNA Clones Encoding a Human β3 Subunit

Sequencing of clones isolated as described in Example III also revealed a clone encoding a human neuronal calcium channel β_3 subunit. This clone has been deposited as plasmid β1.42 (ATCC Accession No. 69048).

To isolate a full-length cDNA clone encoding a complete β_3 subunit, a human hippocampus cDNA library (Stratagene, La Jolla, CA) was screened for hybridization to a 5' *Eco*RI-*Pst*I fragment of the cDNA encoding β_{1-2} using lower stringency hybridization conditions (20% deionized formamide, 200 μg/ml sonicated herring sperm DNA, 5X SSPE, 5X Denhardt's solution, 42° C) and wash conditions. One of the hybridizing clones contained both translation initiation and termination codons

and encodes a complete β_3 subunit designated β_{3-1} (Sequence ID No. 19). *In vitro* transcripts of the cDNA were prepared and injected into *Xenopus* oocytes along with transcripts of the α_{1B-1} and α_{2b} cDNAs using methods similiar to those described in Example IX.D. Two-electrode voltage clamp recordings of the oocytes revealed significant voltage-dependent inward Ba^{2+} currents.

An additional β_3 subunit-encoding clone, designated β_{3-2}, was obtained by screening a human cerebellum cDNA library for hybridization to the nucleic acid amplification product referred to in Example VIII.A. under lower stringency (20% deionized formamide, 200 μg/ml sonicated herring sperm DNA, 5X SSPE, 5X Denhardt's solution, 42° C) hybridization and wash conditions. The 5′ ends of this clone (Sequence ID No. 20, β_{3-2}) and the first β_3 subunit, designated β_{3-1}, (Sequence ID No. 19) differ at their 5′ ends and are splice variants of the β_3 gene.

EXAMPLE IX: RECOMBINANT EXPRESSION OF HUMAN NEURONAL CALCIUM CHANNEL SUBUNIT-ENCODING cDNA AND RNA TRANSCRIPTS IN MAMMALIAN CELLS

A. Recombinant Expression of the Human Neuronal Calcium Channel α_2 subunit cDNA in DG44 Cells

1. Stable transfection of DG44 cells

DG44 cells [dhfr$^-$ Chinese hamster ovary cells; see, *e.g.*, Urlaub, G. *et al.* (1986) *Som. Cell Molec. Genet. 12*:555-566] obtained from Lawrence Chasin at Columbia University were stably transfected by $CaPO_4$ precipitation methods [Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. USA 76*:1373-1376] with pSV2dhfr vector containing the human neuronal calcium channel α_2-subunit cDNA (see Example IV) for polycistronic expression/selection in transfected cells. Transfectants were grown on 10% DMEM medium without hypoxanthine or thymidine in order to select cells that had incorporated the expression vector. Twelve transfectant cell lines were established as indicated by their ability to survive on this medium.

2. Analysis of α_2 subunit cDNA expression in transfected DG44 cells

Total RNA was extracted according to the method of Birnboim [(1988) *Nuc. Acids Res. 16*:1487-1497] from four of the DG44 cell lines that had been stably transfected with pSV2dhfr containing the human neuronal calcium channel α_2 subunit cDNA. RNA (~15 μg per lane) was separated on a 1% agarose formaldehyde gel, transferred to nitrocellulose and hybridized to the random-primed human neuronal calcium channel α_2 cDNA (hybridization: 50% formamide, 5 x SSPE, 5 x Denhardt's, 42° C.; wash :0.2 x SSPE, 0.1% SDS, 65° C.). Northern blot analysis of total RNA from four of the DG44 cell lines that had been stably transfected with pSV2dhfr containing the human neuronal calcium channel α_2 subunit cDNA revealed that one of the four cell lines contained hybridizing mRNA the size expected for the transcript of the α_2 subunit cDNA (5000 nt based on the size of the cDNA) when grown in the presence of 10 mM sodium butyrate for two days. Butyrate nonspecifically induces transcription and is often used for inducing the SV40 early promoter [Gorman, C. and Howard, B. (1983) *Nucleic Acids Res. 11*:1631]. This cell line, 44α_2-9, also produced mRNA species smaller (several species) and larger (6800 nt) than the size expected for the transcript of the α_2 cDNA (5000 nt) that hybridized to the α_2 cDNA-based probe. The 5000- and 6800-nt transcripts produced by this transfectant should contain the entire α_2 subunit coding sequence and therefore should yield a full-length α_2 subunit protein. A weakly hybridizing 8000-nucleotide transcript was present in untransfected and transfected DG44 cells. Apparently, DG44 cells transcribe a calcium channel α_2 subunit or similar gene at low levels. The level of expression of this endogenous α_2 subunit transcript did not appear to be affected by exposing the cells to butyrate before isolation of RNA for northern analysis.

Total protein was extracted from three of the DG44 cell lines that had been stably transfected with pSV2dhfr

containing the human neuronal calcium channel α_2 subunit cDNA. Approximately 10^7 cells were sonicated in 300 μl of a solution containing 50 mM HEPES, 1 mM EDTA, 1 mM PMSF. An equal volume of 2x loading dye [Laemmli, U.K. (1970). *Nature 227*:680] was added to the samples and the protein was subjected to electrophoresis on an 8% polyacrylamide gel and then electrotransferred to nitrocellulose. The nitrocellulose was incubated with polyclonal guinea pig antisera (1:200 dilution) directed against the rabbit skeletal muscle calcium channel α_2 subunit (obtained from K. Campbell, University of Iowa) followed by incubation with [^{125}I]-protein A. The blot was exposed to X-ray film at -70° C. Reduced samples of protein from the transfected cells as well as from untransfected DG44 cells contained immunoreactive protein of the size expected for the α_2 subunit of the human neuronal calcium channel (130-150 kDa). The level of this immunoreactive protein was higher in 44α_2-9 cells that had been grown in the presence of 10 mM sodium butyrate than in 44α_2-9 cells that were grown in the absence of sodium butyrate. These data correlate well with those obtained in northern analyses of total RNA from 44α_2-9 and untransfected DG44 cells. Cell line 44α_2-9 also produced a 110 kD immunoreactive protein that may be either a product of proteolytic degradation of the full-length α_2 subunit or a product of translation of one of the shorter (<5000 nt) mRNAs produced in this cell line that hybridized to the α_2 subunit cDNA probe.

B. Expression of DNA encoding human neuronal calcium channel α_1, α_2 and β_1 subunits in HEK cells

Human embryonic kidney cells (HEK 293 cells) were transiently and stably transfected with human neuronal DNA encoding calcium channel subunits. Individual transfectants were analyzed electrophysiologically for the presence of voltage-activated barium currents and functional recombinant voltage-dependent calcium channels were.

1. Transfection of HEK 293 cells

Separate expression vectors containing DNA encoding human neuronal calcium channel α_{1D}, α_2 and β_1 subunits, plasmids pVDCCIII(A), pHBCaCHα_2A, and pHBCaCHβ_{1a}RBS(A), respectively, were constructed as described in Examples II.A.3, IV.B. and III.B.3., respectively. These three vectors were used to transiently co-transfect HEK 293 cells. For stable transfection of HEK 293 cells, vector pHBCaCHβ_{1b}RBS(A) (Example III.B.3.) was used in place of pHBCaCHβ_{1a}RBS(A) to introduce the DNA encoding the β_1 subunit into the cells along with pVDCCIII(A) and pHBCaCHα_2A.

a. Transient transfection

Expression vectors pVDCCIII(A), pHBCaCHα_2A and pHBCaCHβ_{1a}RBS(A) were used in two sets of transient transfections of HEK 293 cells (ATCC Accession No. CRL1573). In one transfection procedure, HEK 293 cells were transiently cotransfected with the α_1 subunit cDNA expression plasmid, the α_2 subunit cDNA expression plasmid, the β_1 subunit cDNA expression plasmid and plasmid pCMVβgal (Clontech Laboratories, Palo Alto, CA). Plasmid pCMVβgal contains the *lacZ* gene (encoding *E. coli* β-galactosidase) fused to the cytomegalovirus (CMV) promoter and was included in this transfection as a marker gene for monitoring the efficiency of transfection. In the other transfection procedure, HEK 293 cells were transiently co-transfected with the α_1 subunit cDNA expression plasmid pVDCCIII(A) and pCMVβgal. In both transfections, 2-4 x 10^6 HEK 293 cells in a 10-cm tissue culture plate were transiently co-transfected with 5 μg of each of the plasmids included in the experiment according to standard $CaPO_4$ precipitation transfection procedures (Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. USA* 76:1373-1376). The transfectants were analyzed for β-galactosidase expression by direct staining of the product of a reaction involving β-galactosidase and the X-gal substrate [Jones, J.R. (1986) *EMBO* 5:3133-3142] and by measurement of β-galactosidase activity [Miller, J.H. (1972) Experiments in Molecular Genetics, pp.

352-355, Cold Spring Harbor Press]. To evaluate subunit cDNA expression in these transfectants, the cells were analyzed for subunit transcript production (northern analysis), subunit protein production (immunoblot analysis of cell lysates) and functional calcium channel expression (electrophysiological analysis).

b. Stable transfection

HEK 293 cells were transfected using the calcium phosphate transfection procedure [*Current Protocols in Molecular Biology*, Vol. 1, Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)]. Ten-cm plates, each containing one-to-two million HEK 293 cells, were transfected with 1 ml of DNA/calcium phosphate precipitate containing 5 μg pVDCCIII(A), 5 μg pHBCaCHα_2A, 5μg pHBCaCHβ_{1b}RBS(A), 5 μg pCMVBgal and 1 μg pSV2neo (as a selectable marker). After 10-20 days of growth in media containing 500 μg G418, colonies had formed and were isolated using cloning cylinders.

2. Analysis of HEK 293 cells transiently transfected with DNA encoding human neuronal calcium channel subunits

a. Analysis of β-galactosidase expression

Transient transfectants were assayed for β-galactosidase expression by β-galactosidase activity assays (Miller, J.H., (1972) Experiments in Molecular Genetics, pp. 352-355, Cold Spring Harbor Press) of cell lysates (prepared as described in Example VII.A.2) and staining of fixed cells (Jones, J.R. (1986) *EMBO* 5:3133-3142). The results of these assays indicated that approximately 30% of the HEK 293 cells had been transfected.

b. Northern analysis

PolyA+ RNA was isolated using the Invitrogen Fast Trak Kit (InVitrogen, San Diego, CA) from HEK 293 cells transiently transfected with DNA encoding each of the α_1, α_2 and β_1 subunits and the *lacZ* gene or the α_1 subunit and the *lacZ* gene. The RNA was subjected to electrophoresis on an agarose gel and transferred to nitrocellulose. The nitrocellulose was then hybridized with one or more of the following radiolabeled

probes: the *lacZ* gene, human neuronal calcium channel α_{1D} subunit-encoding cDNA, human neuronal calcium channel α_2 subunit-encoding cDNA or human neuronal calcium channel β_1 subunit-encoding cDNA. Two transcripts that hybridized with the α_1 subunit-encoding cDNA were detected in HEK 293 cells transfected with the DNA encoding the α_1, α_2, and β_1 subunits and the *lacZ* gene as well as in HEK 293 cells transfected with the α_1 subunit cDNA and the *lacZ* gene. One mRNA species was the size expected for the transcript of the α_1 subunit cDNA (8000 nucleotides). The second RNA species was smaller (4000 nucleotides) than the size expected for this transcript. RNA of the size expected for the transcript of the *lacZ* gene was detected in cells transfected with the α_1, α_2 and β_1 subunit-encoding cDNA and the *lacZ* gene and in cells transfected with the α_1 subunit cDNA and the *lacZ* gene by hybridization to the *lacZ* gene sequence.

RNA from cells transfected with the α_1, α_2 and β_1 subunit-encoding cDNA and the *lacZ* gene was also hybridized with the α_2 and β_1 subunit cDNA probes. Two mRNA species hybridized to the α_2 subunit cDNA probe. One species was the size expected for the transcript of the α_2 subunit cDNA (4000 nucleotides). The other species was larger (6000 nucleotides) than the expected size of this transcript. Multiple RNA species in the cells co-transfected with α_1, α_2 and β_1 subunit-encoding cDNA and the *lacZ* gene hybridized to the β_1 subunit cDNA probe. Multiple β subunit transcripts of varying sizes were produced since the β subunit cDNA expression vector contains two potential polyA$^+$ addition sites.

c. Electrophysiological analysis

Individual transiently transfected HEK 293 cells were assayed for the presence of voltage-dependent barium currents using the whole-cell variant of the patch clamp technique [Hamill et al. (1981). *Pflugers Arch. 391*:85-100]. HEK 293 cells transiently transfected with pCMVβgal only were assayed for barium currents as a negative control in these xperiments. The cells were placed in a bathing solution that

contained barium ions to serve as the current carrier. Choline chloride, instead of NaCl or KCl, was used as the major salt component of the bath solution to eliminate currents through sodium and potassium channels. The bathing solution contained 1 mM $MgCl_2$ and was buffered at pH 7.3 with 10 mM HEPES (pH adjusted with sodium or tetraethylammonium hydroxide). Patch pipettes were filled with a solution containing 135 mM CsCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM EGTA, 4 mM ATP and 10 mM HEPES (pH adjusted to 7.3 with tetraethylammonium hydroxide). Cesium and tetraethylammonium ions block most types of potassium channels. Pipettes were coated with Sylgard (Dow-Corning, Midland, MI) and had resistances of 1-4 megohm. Currents were measured through a 500 megohm headstage resistor with the Axopatch IC (Axon Instruments, Foster City, CA) amplifier, interfaced with a Labmaster (Scientific Solutions, Solon, OH) data acquisition board in an IBM-compatible PC. PClamp (Axon Instruments) was used to generate voltage commands and acquire data. Data were analyzed with pClamp or Quattro Professional (Borland International, Scotts Valley, CA) programs.

To apply drugs, "puffer" pipettes positioned within several micrometers of the cell under study were used to apply solutions by pressure application. The drugs used for pharmacological characterization were dissolved in a solution identical to the bathing solution. Samples of a 10 mM stock solution of Bay K 8644 (RBI, Natick, MA), which was prepared in DMSO, were diluted to a final concentration of 1 μM in 15 mM Ba^{2+}-containing bath solution before they were applied.

Twenty-one negative control HEK 293 cells (transiently transfected with the *lacZ* gene expression vector pCMVβgal only) were analyzed by the whole-cell variant of the patch clamp method for recording currents. Only one cell displayed a discernable inward barium current; this current was not affected by the presence of 1 μM Bay K 8644. In addition, application of Bay K 8644 to four cells that did not display Ba^{2+} currents did not result in the appearance of any currents.

Two days after transient transfection of HEK 293 cells with α_1, α_2 and β_1 subunit-encoding cDNA and the *lacZ* gene, individual transfectants were assayed for voltage-dependent barium currents. The currents in nine transfectants were recorded. Because the efficiency of transfection of one cell can vary from the efficiency of transfection of another cell, the degree of expression of heterologous proteins in individual transfectants varies and some cells do not incorporate or express the foreign DNA. Inward barium currents were detected in two of these nine transfectants. In these assays, the holding potential of the membrane was -90 mV. The membrane was depolarized in a series of voltage steps to different test potentials and the current in the presence and absence of 1 μM Bay K 8644 was recorded. The inward barium current was significantly enhanced in magnitude by the addition of Bay K 8644. The largest inward barium current (~160 pA) was recorded when the membrane was depolarized to 0 mV in the presence of 1 μM Bay K 8644. A comparison of the I-V curves, generated by plotting the largest current recorded after each depolarization versus the depolarization voltage, corresponding to recordings conducted in the absence and presence of Bay K 8644 illustrated the enhancement of the voltage-activated current in the presence of Bay K 8644.

Pronounced tail currents were detected in the tracings of currents generated in the presence of Bay K 8644 in HEK 293 cells transfected with α_1, α_2 and β_1 subunit-encoding cDNA and the *lacZ* gene, indicating that the recombinant calcium channels responsible for the voltage-activated barium currents recorded in this transfected appear to be DHP-sensitive.

The second of the two transfected cells that displayed inward barium currents expressed a ~50 pA current when the membrane was depolarized from -90 mV. This current was nearly completely blocked by 200 μM cadmium, an established calcium channel blocker.

Ten cells that were transiently transfected with the DNA encoding the α_1 subunit and the *lacZ* gene were analyzed by

whole-cell patch clamp methods two days after transfection. One of these cells displayed a 30 pA inward barium current. This current amplified 2-fold in the presence of 1 μM Bay K 8644. Furthermore, small tail currents were detected in the presence of Bay K 8644. These data indicate that expression of the human neuronal calcium channel α_{1D} subunit-encoding cDNA in HEK 293 yields a functional DHP-sensitive calcium channel.

3. Analysis of HEK 293 cells stably transfected with DNA encoding human neuronal calcium channel subunits

Individual stably transfected HEK 293 cells were assayed electrophysiologically for the presence of voltage-dependent barium currents as described for electrophysiological analysis of transiently transfected HEK 293 cells (see Example VII.B.2.c). In an effort to maximize calcium channel activity via cyclic-AMP-dependent kinase-mediated phosphorylation [Pelzer, et al. (1990) *Rev. Physiol. Biochem. Pharmacol.* 114:107-207], cAMP (Na salt, 250 μM) was added to the pipet solution and forskolin (10 μM) was added to the bath solution in some of the recordings. Qualitatively similar results were obtained whether these compounds were present or not.

Barium currents were recorded from stably transfected cells in the absence and presence of Bay K 8644 (1 μM). When the cell was depolarized to -10 mV from a holding potential of -90 mV in the absence of Bay K 8644, a current of approximately 35pA with a rapidly deactivating tail current was recorded. During application of Bay K 8644, an identical depolarizing protocol elicited a current of approximately 75 pA, accompanied by an augmented and prolonged tail current. The peak magnitude of currents recorded from this same cell as a function of a series of depolarizing voltages were assessed. The responses in the presence of Bay K 8644 not only increased, but the entire current-voltage relation shifted about -10 mV. Thus, three typical hallmarks of Bay K 8644 action, namely increased current magnitude, prolonged tail currents, and negatively shifted activation voltage, were

observed, clearly indicating the expression of a DHP-sensitive calcium channel in these stably transfected cells. No such effects of Bay K 8644 were observed in untransfected HEK 293 cells, either with or without cAMP or forskolin.

C. Use of pCMV-based vectors and pcDNA1-based vectors for expression of DNA encoding human neuronal calcium channel subunits

1. Preparation of constructs

Additional expression vectors were constructed using pCMV. The full-length α_{1D} cDNA from pVDCCIII(A) (see Example II.A.3.d), the full-length α_2 cDNA, contained on a 3600 bp *Eco*RI fragment from HBCaCHα_2 (see Example IV.B) and a full-length β_1 subunit cDNA from pHBCaCHβ_{1b}RBS(A) (see Example III.B.3) were separately subcloned into plasmid pCMVβgal. Plasmid pCMVβgal was digested with *Not*I to remove the *lacZ* gene. The remaining vector portion of the plasmid, referred to as pCMV, was blunt-ended at the *Not*I sites. The full-length α_2-encoding DNA and β_1-encoding DNA, contained on separate *Eco*RI fragments, were isolated, blunt-ended and separately ligated to the blunt-ended vector fragment of pCMV locating the cDNAs between the CMV promoter and SV40 polyadenylation sites in pCMV. To ligate the α_{1D}-encoding cDNA with pCMV, the restriction sites in the polylinkers immediately 5' of the CMV promoter and immediately 3' of the SV40 polyadenylation site were removed from pCMV. A polylinker was added at the *Not*I site. The polylinker had the following sequence of restriction enzyme recognition sites:

GGCCGC CG	*Eco*RI site	*Sal*I site	*Pst*I site	*Eco*RV site	*Hin*dIII site	*Xba*II site	GT CACCGG ↑
*Not*I							Destroys *Not*

The α_{1D}-encoding DNA, isolated as a *Bam*HI/*Xho*I fragment from pVDCCIII(A), was then ligated to *Xba*II/*Sal*I-digested pCMV to place it between the CMV promoter and SV40 polyadenylation site.

Plasmid pCMV contains the CMV promoter as does pcDNA1, but differs from pcDNA1 in the location of splice donor/splice acceptor sites relative to the inserted subunit-encoding DNA. After inserting the subunit-encoding DNA into pCMV, the splice donor/splice acceptor sites are located 3′ of the CMV promoter and 5′ of the subunit-encoding DNA start codon. After inserting the subunit-encoding DNA into pcDNA1, the splice donor/splice acceptor sites are located 3′ of the subunit cDNA stop codon.

2. Transfection of HEK 293 cells

HEK 293 cells were transiently co-transfected with the α_{1D}, α_2 and β_1 subunit-encoding DNA in pCMV or with the α_{1D}, α_2 and β subunit-encoding DNA in pcDNA1 (vectors pVDCCIII(A), pHBCaCHα_2A and pHBCaCHβ_{1b}RBS(A), respectively), as described in Example VII.B.1.a. Plasmid pCMVβgal was included in each transfection as a measure of transfection efficiency. The results of β-galactosidase assays of the transfectants (see Example VII.B.2.), indicated that HEK 293 cells were transfected equally efficiently with pCMV- and pcDNA1-based plasmids. The pcDNA1-based plasmids, however, are presently preferred for expression of calcium channel receptors.

D. Expression in Xenopus laevis oöcytes of RNA encoding human neuronal calcium channel subunits

Various combinations of the transcripts of DNA encoding the human neuronal α_{1D}, α_2 and β_1 subunits prepared *in vitro* were injected into *Xenopus laevis* oöcytes. Those injected with combinations that included a_{1D} exhibited voltage-activated barium currents.

1. Preparation of transcripts

Transcripts encoding the human neuronal calcium channel α_{1D}, α_2 and β_1 subunits were synthesized according to the instructions of the mCAP mRNA CAPPING KIT (Strategene, La Jolla, CA catalog #200350). Plasmids pVDCC III.RBS(A), containing pcDNA1 and the α_{1D} cDNA that begins with a ribosome binding site and the eighth ATG codon of the coding sequence (see Example III.A.3.d), plasmid pHBCaCHα_1A containing pcDNA1 and an α_2 subunit cDNA (see Example IV), and plasmid pHBCaCHβ_{1b}RBS(A) containing pcDNA1 and the β_1 DNA lacking intron sequence and containing a ribosome binding site (see Example III), were linearized by restriction digestion. The α_{1D} cDNA- and α_2 subunit-encoding plasmids were digested with *Xho*I, and the β_1 subunit- encoding plasmid was digested with *Eco*RV. The DNA insert was transcribed with T7 RNA polymerase.

2. Injection of oöcytes

Xenopus laevis oöcytes were isolated and defolliculated by collagenase treatment and maintained in 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6, 20 μg/ml ampicillin and 25 μg/ml streptomycin at 19-25°C for 2 to 5 days after injection and prior to recording. For each transcript that was injected into the oöcyte, 6 ng of the specific mRNA was injected per cell in a total volume of 50 nl.

3. Intracellular voltage recordings

Injected oöcytes were examined for voltage-dependent barium currents using two-electrode voltage clamp methods [Dascal, N. (1987) *CRC Crit. Rev. Biochem.* *22*:317]. The pClamp (Axon Instruments) software package was used in conjunction with a Labmaster 125 kHz data acquisition interface to generate voltage commands and to acquire and analyze data. Quattro Professional was also used in this analysis. Current signals were digitized at 1-5 kHz, and filtered appropriately. The bath solution contained of the following: 40 mM $BaCl_2$, 36 mM tetraethylammonium chloride

(TEA-Cl), 2 mM KCl, 5 mM 4-aminopyridine, 0.15 mM niflumic acid, 5 mM HEPES, pH 7.6.

a. Electrophysiological analysis of oöcytes injected with transcripts encoding the human neuronal calcium channel α_1, α_2 and β_1-subunits

Uninjected oöcytes were examined by two-electrode voltage clamp methods and a very small (25 nA) endogenous inward Ba^{2+} current was detected in only one of seven analyzed cells.

Oöcytes coinjected with α_{1D}, α_2 and β_1 subunit transcripts expressed sustained inward barium currents upon depolarization of the membrane from a holding potential of -90 mV or -50 mV (154 ± 129 nA, n=21). These currents typically showed little inactivation when test pulses ranging from 140 to 700 msec. were administered. Depolarization to a series of voltages revealed currents that first appeared at approximately -30 mV and peaked at approximately 0 mV.

Application of the DHP Bay K 8644 increased the magnitude of the currents, prolonged the tail currents present upon repolarization of the cell and induced a hyperpolarizing shift in current activation. Bay K 8644 was prepared fresh from a stock solution in DMSO and introduced as a 10x concentrate directly into the 60 μl bath while the perfusion pump was turned off. The DMSO concentration of the final diluted drug solutions in contact with the cell never exceeded 0.1%. Control experiments showed that 0.1% DMSO had no effect on membrane currents.

Application of the DHP antagonist nifedipine (stock solution prepared in DMSO and applied to the cell as described for application of Bay K 8644) blocked a substantial fraction (91 ± 6%, n=7) of the inward barium current in oöcytes coinjected with transcripts of the α_{1D}, α_2 and β_1 subunits. A residual inactivating component of the inward barium current typically remained after nifedipine application. The inward barium current was blocked completely by 50 μM Cd^{2+}, but only approximately 15% by 100 μM Ni^{2+}.

The effect of ωCgTX on the inward barium currents in oöcytes co-injected with transcripts of the α_{1D}, α_2, and β_1 subunits was investigated. ωCgTX (Bachem, Inc., Torrance CA) was prepared in the 15 mM $BaCl_2$ bath solution plus 0.1% cytochrome C (Sigma) to serve as a carrier protein. Control experiments showed that cytochrome C had no effect on currents. A series of voltage pulses from a -90 mV holding potential to 0 mV were recorded at 20 msec. intervals. To reduce the inhibition of ωCgTX binding by divalent cations, recordings were made in 15 mM $BaCl_2$, 73.5 mM tetraethylammonium chloride, and the remaining ingredients identical to the 40 mM Ba^{2+} recording solution. Bay K 8644 was applied to the cell prior to addition to ωCgTX in order to determine the effect of ωCgTX on the DHP-sensitive current component that was distinguished by the prolonged tail currents. The inward barium current was blocked weakly (54 ± 29%, n=7) and reversibly by relatively high concentrations (10-15 μM) of ωCgTX. The test currents and the accompanying tail currents were blocked progressively within two to three minutes after application of ωCgTX, but both recovered partially as the ωCgTX was flushed from the bath.

b. Analysis of oöcytes injected with only a transcripts encoding the human neuronal calcium channel α_{1D} or transcripts encoding an α_{1D} and other subunits

The contribution of the α_2 and β_1 subunits to the inward barium current in oöcytes injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits was assessed by expression of the α_{1D} subunit alone or in combination with either the β_1 subunit or the α_2 subunit. In oöcytes injected with only the transcript of a α_{1D} cDNA, no Ba^{2+} currents were detected (n=3). In oöcytes injected with transcripts of α_{1D} and β_1 cDNAs, small (108 ± 39 nA) Ba^{2+} currents were detected upon depolarization of the membrane from a holding potential of -90 mV that resembled the currents observed in cells injected with transcripts of α_{1D}, α_2 and β_1 cDNAs, although the magnitude of

the current was less. In two of the four oöcytes injected with transcripts of the α_{1D}-encoding and β_1-encoding DNA, the Ba^{2+} currents exhibited a sensitivity to Bay K 8644 that was similar to the Bay K 8644 sensitivity of Ba^{2+} currents expressed in oöcytes injected with transcripts encoding the α_{1D} α_1-, α_2- and β_1 subunits.

Three of five oöcytes injected with transcripts encoding the α_{1D} and α_2 subunits exhibited very small Ba^{2+} currents (15-30 nA) upon depolarization of the membrane from a holding potential of -90 mV. These barium currents showed little or no response to Bay K 8644.

c. Analysis of oöcytes injected with transcripts encoding the human neuronal calcium channel α_2 and/or β_1 subunit

To evaluate the contribution of the α_{1D} α_1-subunit to the inward barium currents detected in oöcytes co-injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits, oöcytes injected with transcripts encoding the human neuronal calcium channel α_2 and/or β_1 subunits were assayed for barium currents. Oöcytes injected with transcripts encoding the α_2 subunit displayed no detectable inward barium currents (n=5). Oöcytes injected with transcripts encoding a β_1 subunit displayed measurable (54 ± 23 nA, n=5) inward barium currents upon depolarization and oöcytes injected with transcripts encoding the α_2 and β_1 subunits displayed inward barium currents that were approximately 50% larger (80 ± 61 nA, n=18) than those detected in oöcytes injected with transcripts of the β_1-encoding DNA only.

The inward barium currents in oöcytes injected with transcripts encoding the β_1 subunit or α_2 and β_1 subunits typically were first observed when the membrane was depolarized to -30 mV from a holding potential of -90 mV and peaked when the membrane was depolarized to 10 to 20 mV. Macroscopically, the currents in oöcytes injected with transcripts encoding the α_2 and β_1 subunits or with transcripts encoding the β_1 subunit were indistinguishable. In contrast to the currents in oöcytes co-injected with transcripts of α_{1D},

α_2 and β_1 subunit cDNAs, these currents showed a significant inactivation during the test pulse and a strong sensitivity to the holding potential. The inward barium currents in oöcytes co-injected with transcripts encoding the α_2 and β_1 subunits usually inactivated to 10-60% of the peak magnitude during a 140-msec pulse and were significantly more sensitive to holding potential than those in oöcytes co-injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits. Changing the holding potential of the membranes of oöcytes co-injected with transcripts encoding the α_2 and β_1 subunits from -90 to -50 mV resulted in an approximately 81% (n=11) reduction in the magnitude of the inward barium current of these cells. In contrast, the inward barium current measured in oöcytes co-injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits were reduced approximately 24% (n=11) when the holding potential was changed from -90 to -50 mV.

The inward barium currents detected in oöcytes injected with transcripts encoding the α_2 and β_1 subunits were pharmacologically distinct from those observed in oöcytes co-injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits. Oöcytes injected with transcripts encoding the α_2 and β_1 subunits displayed inward barium currents that were insensitive to Bay K 8644 (n=11). Nifedipine sensitivity was difficult to measure because of the holding potential sensitivity of nifedipine and the current observed in oöcytes injected with transcripts encoding the α_2 and β_1 subunits. Nevertheless, two oöcytes that were co-injected with transcripts encoding the α_2 and β_1 subunits displayed measurable (25 to 45 nA) inward barium currents when depolarized from a holding potential of -50 mV. These currents were insensitive to nifedipine (5 to 10 μM). The inward barium currents in oöcytes injected with transcripts encoding the α_2 and β_1 subunits showed the same sensitivity to heavy metals as the currents detected in oöcytes injected with transcripts encoding the α_{1D}, α_2 and β_1 subunits.

The inward barium current detected in oöcytes injected with transcripts encoding the human neuronal α_2 and β_1 subunits has pharmacological and biophysical properties that resemble calcium currents in uninjected *Xenopus* oöcytes. Because the amino acids of this human neuronal calcium channel β_1 subunit lack hydrophobic segments capable of forming transmembrane domains, it is unlikely that recombinant β_1 subunits alone can form an ion channel. It is more probable that a homologous endogenous α_1 subunit exists in oöcytes and that the activity mediated by such an α_1 subunit is enhanced by expression of a human neuronal β_1 subunit.

E. Expression of DNA encoding human neuronal calcium channel α_{1B}, α_{2B} and β_{1-2} subunits in HEK cells

1. Transfection of HEK cells

The transient expression of the human neuronal α_{1B-1}, α_{2b} and β_{1-2} subunits was studied in HEK293 cells. The HEK293 cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium (Gibco) containing 5% defined-supplemented bovine calf serum (Hyclone) plus penicillin G (100 U/ml) and steptomycin sulfate (100 µg/ml). HEK293 cell transfections were mediated by calcium phosphate as described above. Transfected cells were examined for inward Ba^{2+} currents (I_{Ba}) mediated by voltage-dependent Ca^{2+} channels.

Cells were transfected (2 x 10^6 per polylysine-coated plate. Standard transfections (10-cm dish) contained 8 µg of pcDNAα_{1B-1}, 5 µg of pHBCaCHα_2A, 2 µg pHBCaCHβ_{1b}RBS(A) (see, Examples II.A.3, IV.B. and III) and 2 µg of CMVβ (Clontech) β-glactosidase expression plasmid, and pUC18 to maintain a constant mass of 20 µg/ml. Cells were analyzed 48 to 72 hours after transfection. Transfection efficiencies (±10%), which were determined by in situ histochemical staining for β-galactosidase activity (Sanes *et al.* (1986) *EMBO J.*, 5:3133), generally were greater than 50%.

2. Electrophysiological analysis of transfectant currents

a. Materials and m thods

Properties of recombinantly expressed Ca^{2+} channels were studied by whole cell patch-clamp techniques. Recordings were performed on transfected HEK293 cells 2 to 3 days after transfection. Cells were plated at 100,000 to 300,000 cells per polylysine-coated, 35-mm tissue culture dishes (Falcon, Oxnard, CA) 24 hours before recordings. Cells were perfused with 15 mM $BaCl_2$, 125 mM choline chloride, 1 mM $MgCl_2$, and 10 mM Hepes (pH = 7.3) adjusted with tetraethylammonium hydroxide (bath solution). Pipettes were filled with 135 mM CsCl, 10 mM EGTA, 10 mM Hepes, 4 mM Mg-adenosine triphosphate (pH = 7.5) adjusted with tetraethylammonium hydroxide. Sylgard (Dow-Corning, Midland, MI)-coated, fire-polished, and filled pipettes had resistances of 1 to 2 megohm before gigohm seals were established to cells.

Bay K 8644 and nifedipine (Research Biochemicals, Natick, MA) were prepared from stock solutions (in dimethyl sulfoxide) and diluted into the bath solution. The dimethyl sulfoxide concentration in the final drug solutions in contact with the cells never exceeded 0.1%. Control experiments showed that 0.1% dimethyl sulfoxide had no efect on membrane currents. ωCgTX (Bachem, Inc., Torrance CA) was prepared in the 15 mM $BaCl_2$ bath solution plus 0.1% cytochrome C (Sigma, St. Louis MO) to serve as a carrier protein. Control experiments showed that cytochrome C had no effect on currents. These drugs were dissolved in bath solution, and continuously applied by means of puffer pipettes as required for a given experiment. Recordings were performed at room temperature (22° to 25°C). Series resistance compensation (70 to 85%) was employed to minimize voltage error that resulted from pipette access resistance, typically 2 to 3.5 megohm. Current signals were filtered (-3 dB, 4-pole Bessel) at a frequency of 1/4 to 1/5 the sampling rate, which ranged from 0.5 to 3 kHz. Voltage commands were generated and data were acquired with CLAMPEX (pClamp, Axon Instruments, Foster City, CA). All reported data are corrected for linear leak and capacitive

components. Exponential fitting of currents was performed with CLAMPFIT (Axon Instruments, Foster City, CA).

b. Results

Transfectants were examined for inward Ba^{2+} currents (I_{Ba}). Cells cotransfected with DNA encoding α_{1B-1}, α_{2b}, and β_{1-2} subunits expressed high-voltage-activated Ca^{2+} channels. I_{Ba} first appeared when the membrane was depolarized from a holding potential of -90 mV to -20 mV and peaked in magnitude at 10 mV. Thirty-nine of 95 cells (12 independent transfections) had I_{Ba} that ranged from 30 to 2700 pA, with a mean of 433 pA. The mean current density was 26 pA/pF, and the highest density was 150 pA/pF. The I_{Ba} typically increased by 2- to 20-fold during the first 5 minutes of recording. Repeated depolarizations during long records often revealed rundown of I_{Ba} usually not exceeding 20% within 10 min. I_{Ba} typically activated within 10 ms and inactivated with both a fast time constant ranging from 46 to 105 ms and a slow time constant ranging from 291 to 453 ms (n = 3). Inactivation showed a complex voltage dependence, such that I_{Ba} elicited at ≥20 mV inactivated more slowly than I_{Ba} elicited at lower test voltages, possibly a result of an increase in the magnitude of slow compared to fast inactivation components at higher test voltages.

Recombinant $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ channels were sensitive to holding potential. Steady-state inactivation of I_{Ba}, measured after a 30- to 60-s conditioning at various holding potentials, was approximately 50% at holding potential between -60 and -70 mV and approximately 90% at -40 mV. Recovery of I_{Ba} from inactivation was usually incomplete, measuring 55 to 75% of the original magnitude within 1 min. after the holding potential was returned to more negative potentials, possibly indicating some rundown or a slow recovery rate.

Recombinant $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ channels were also blocked irreversibly by ω-CgTx concentrations ranging from 0.5 to 10 μM during the time scale of the experiments. Application of 5 μM toxin (n = 7) blocked the activity completely within

2 min., and no recovery of I_{Ba} was observed after washing ω-CgTx from the bath for up to 15 min. d^{2+} blockage (50 μM) was rapid, complete, and reversible; the DHPs Bay K 8644 (1 μM; n = 4) or nifedipine (5 μM; n = 3) had no discernable effect.

Cells cotransfected with DNA encoding α_{1B-1}, α_{2b}, and β_{1-2} subunits predominantly displayed a single class of saturable, high-affinity ω-CgTx binding sites. The determined dissociation constant (K_d) value was 54.6 ± 14.5 pM (n = 4). Cells transfected with the vector containing only β-galactosidase-encoding DNA or $\alpha_{2b}\beta$-encoding DNA showed no specific binding. The binding capacity (B_{max}) of the $\alpha_{1B-1}\alpha_{2b}\beta$-transfected cells was 28,710 ± 11,950 sites per cell (n = 4).

These results demonstrate that $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$-transfected cells express high-voltage-activated, inactivating Ca^{2+} channel activity that is irreversibly blocked by ω-CgTx, insensitive to DHPs, and sensitive to holding potential. The activation and inactivation kinetics and voltage sensitivity of the channel formed in these cells are generally consistent with previous characterizations of neuronal N-type Ca^{2+} channels.

F. Expression of DNA encoding human neuronal calcium channel α_{1B-1}, α_{1B-2}, α_{2B}, β_{1-2} and β_{1-3} subunits in HEK cells

Significant Ba^{2+} currents were not detected in untransfected HEK293 cells. Furthermore, untransfected HEK293 cells do not express detectable ω-CgTx GVIA binding sites.

In order to approximate the expression of a homogeneous population of trimeric α_{1B}, α_{2b} and $ß_1$ protein complexes in transfected HEK293 cells, the α_{1B}, α_{2b} and $ß_1$ expression levels were altered. The efficiency of expression and assembly of channel complexes at the cell surface were optimized by adjusting the molar ratio of α_{1B}, α_{2b} and $ß_1$ expression plasmids used in the transfections. The transfectants were analyzed for mRNA levels, ω-CgTx GVIA binding and Ca^{2+} channel current density in order to determine near optimal channel expression in the absence of immunological reagents for evaluating

protein expression. Higher molar ratios of α_{2b} appeared to increase calcium channel activity.

1. Transfections

HEK293 cells were maintained in DMEM (Gibco #320-1965AJ), 5.5% Defined/Supplemented bovine calf serum (Hyclone #A-2151-L), 100 U/ml penicillin G and 100 µg/ml streptomycin. Ca^{2+}-phosphate based transient transfections were performed and analyzed as described above. Cells were co-transfected with either 8 µg pcDNA1α_{1B-1} (described in Example II.C), 5 µg pHBCaCHα_2A (see, Example IV.B.), 2 µg pHBCaCHβ_{1b}RBS(A) (β_{1-2} expression plasmid; see Examples III.A. and IX.E.), and 2 µg pCMVβ-gal [Clontech, Palo Alto, CA] (2:1.8:1 molar ratio of Ca^{2+} channel subunit expression plasmids) or with 3 µg pcDNA1α_{1B-1} or pcDNA1α_{1B-2}, 11.25 µg pHBCaCHα_2A, 0.75 or 1.0 µg pHBCaCHβ_{1b}RBS(A) or pcDNA1β_{1-3} and 2 µg pCMVβ-gal (2:10.9:1 molar ratio of Ca^{2+} channel subunit expression plasmids). Plasmid pCMVβ-gal, a ß-galactosidase expression plasmid, was included in the transfections as a marker to permit transfection efficiency estimates by histochemical staining. When less than three subunits were expressed, pCMVPL2, a pCMV promoter-containing vector that lacks a cDNA insert, was substituted to maintain equal moles of pCMV-based DNA in the transfection. pUC18 DNA was used to maintain the total mass of DNA in the transfection at 20 µg/plate.

RNA from the transfected cells was analyzed by Northern blot analysis for calcium channel subunit mRNA expression using random primed ^{32}P-labeled subunit specific probes. HEK293 cells co-transfected with α_{1B-1}, α_{2b} and β_{1-2} expression plasmids (8, 5 and 2 µg, respectively; molar ratio = 2:1.8:1) did not express equivalent levels of each Ca^{2+} channel subunit mRNA. Relatively high levels of α_{1B-1} and β_{1-2} mRNAs were expressed, but significantly lower levels of α_{2b} mRNA were expressed. Based on autoradiograph exposures required to produce equivalent signals for all three mRNAs, α_{2b} transcript levels were estimated to be 5 to 10 times lower than α_{1B-1} and

β_{1-2} transcript levels. Untransfected HEK293 cells did not express detectable levels of α_{1B-1}, α_{2b}, or β_{1-2} mRNAs.

To achieve equivalent Ca^{2+} channel subunit mRNA expression levels, a series of transfections was performed with various amounts of α_{1B-1}, α_{2b} and β_{1-2} expression plasmids. Because the α_{1B-1} and ß$_{1-2}$ mRNAs were expressed at very high levels compared to α_{2b} mRNA, the mass of α_{1B-1} and ß$_{1-2}$ plasmids was lowered and the mass of α_{2b} plasmid was increased in the transfection experiments. Co-transfection with 3, 11.25 and 0.75 μg of α_{1B-1}, α_{2b} and β_{1-2} expression plasmids, respectively (molar ratio = 2:10.9:1), approached equivalent expression levels of each Ca^{2+} channel subunit mRNA. The relative molar quantity of α_{2b} expression plasmid to α_{1B-1} and ß$_{1-2}$ expression plasmids was increased 6-fold. The mass of α_{1B-1} and β_{1-2} plasmids in the transfection was *decreased* 2.67-fold and the mass of α_{2b} plasmid was *increased* 2.25-fold. The 6-fold molar increase of α_{2b} relative to α_{1B-1} and ß$_{1-2}$ required to achieve near equal abundance mRNA levels is consistent with the previous 5- to 10-fold lower estimate of relative α_{2b} mRNA abundance. ω-CgTx GVIA binding to cells transfected with various amounts of expression plasmids indicated that the 3, 11.25 and 0.75 μg of α_{1B-1}, α_{2b} and β_{1-2} plasmids, respectively, improved the level of cell surface expression of channel complexes. Further increases in the mass of α_{2b} and ß$_{1-2}$ expression plasmids while α_{1B-1} was held constant, and alterations in the mass of the α_{1B-1} expression plasmid while α_{2b} and ß$_{1-2}$ were held constant, indicated that the cell surface expression of ω-CgTx GVIA binding sites per cell was nearly optimal. All subsequent transfections were performed with 3, 11.25 and 0.75 μg or 1.0 μg of α_{1B-1} or α_{1B-2}, α_{2b} and β_{1-2} or β_{1-3} expression plasmids, respectively.

2. ^{125}I-ω-CgTx GVIA binding to transfected cells

Statistical analysis of the K_d and B_{max} values was performed using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple pairwise comparisons ($p \leq 0.05$).

Combinations of human voltage-dependent Ca^{2+} channel subunits, α_{1B-1}, α_{1B-2}, α_{2b}, β_{1-2} and β_{1-3}, were analyzed for saturation binding of ^{125}I-ω-CgTx GVIA. About 200,000 cells were used per assay, except for the α_{1B-1}, α_{1B-2}, $\alpha_{1B-1}\alpha_{2b}$ and $\alpha_{1B-2}\alpha_{2b}$ combinations which were assayed with 1 x 10^6 cells per tube The transfected cells displayed a single-class of saturable, high-affinity binding sites. The values for the dissociation constants (K_d) and binding capacities (B_{max}) were determined for the different combinations. The results are summarized as follows:

Subunit Combination	K_d (pM)	B_{max} (sites/cell)
$\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$	54.9 ± 11.1 (n=4)	45,324 ± 15,606
$\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$	53.2 ± 3.6 (n=3)	91,004 ± 37,654
$\alpha_{1B-1}\beta_{1-2}$	17.9 ± 1.9 (n=3)	5,756 ± 2,163
$\alpha_{1B-1}\beta_{1-3}$	17.9 ± 1.6 (n=3)	8,729 ± 2,980
$\alpha_{1B-1}\alpha_{2b}$	84.6 ± 15.3 (n=3)	2,256 ± 356
α_{1B-1}	31.7 ± 4.2 (n=3)	757 ± 128
$\alpha_{1B-2}\alpha_{2b}\beta_{1-2}$	53.0 ± 4.8 (n=3)	19,371 ± 3,798
$\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$	44.3 ± 8.1 (n=3)	37,652 ± 8,129
$\alpha_{1B-2}\beta_{1-2}$	16.4 ± 1.2 (n=3)	2,126 ± 412
$\alpha_{1B-2}\beta_{1-3}$	22.2 ± 5.8 (n=3)	2,944 ± 1,168
$\alpha_{1B-2}\alpha_{2b}$	N.D.* (n=3)	N.D.
α_{1B-2}	N.D.	N.D.

* N.D. = not detectable

Cells transfected with subunit combinations lacking either the α_{1B-1} or the α_{1B-2} subunit did not exhibit any detectable ^{125}I-ω-CgTx GVIA binding (≤ 600 sites/cell). ^{125}I-ω-CgTx GVIA binding to HEK293 cells transfected with α_{1B-2} alone or $\alpha_{1B-2}\alpha_{2b}$ was too low for reliable Scatchard analysis of the data. Comparison of the K_d and B_{max} values revealed several relationships between specific combinations of subunits and the binding affinities and capacities of the transfected cells. In cells transfected with all three subunits, ($\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$-, $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$-, $\alpha_{1B-2}\alpha_{2b}\beta_{1-2}$-, or $\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$-transfectants) the K_d values were indistinguishable ($p>0.05$), ranging from 44.3

± 8.1 pM to 54.9 ± 11.1 pM. In cells transfected with two-subunit combinations lacking the α_{2b} subunit ($\alpha_{1B-1}\beta_{1-2}$, $\alpha_{1B-1}\beta_{1-3}$, $\alpha_{1B-2}\beta_{1-2}$ or $\alpha_{1B-2}\beta_{1-3}$) the K_d values were significantly lower than the three-subunit combinations ($p<0.01$), ranging from 16.4 ± 1.2 to 22.2 ± 5.8 pM. Cells transfected with only the α_{1B-1} subunit had a K_d value of 31.7 ± 4.2 pM, a value that was not different from the two-subunit combinations lacking α_{2b} ($p<0.05$). As with the comparison between the four $\alpha_{1B}\alpha_{2b}\beta_1$ *versus* $\alpha_{1B}\beta_1$ combinations, when the α_{1B-1} was co-expressed with α_{2b}, the K_d increased significantly ($p<0.05$) from 31.7 ± 4.2 to 84.6 ± 5.3 pM. These data demonstrate that co-expression of the α_{2b} subunit with α_{1B-1}, $\alpha_{1B-1}\beta_{1-2}$, $\alpha_{1B-1}\beta_{1-3}$, $\alpha_{1B-2}\beta_{1-2}$ or $\alpha_{1B-2}\beta_{1-3}$ subunit combinations results in lower binding affinity of the cell surface receptors for ^{125}I-ω-CgTx GVIA. The B_{max} values of cells transfected with various subunit combinations also differed considerably. Cells transfected with the α_{1B-1} subunit alone expressed a low but detectable number of binding sites (approximately 750 binding sites/cell). When the α_{1B-1} subunit was co-expressed with the α_{2b} subunit, the binding capacity increased approximately three-fold while co-expression of a β_{1-2} or β_{1-3} subunit with α_{1B-1} resulted in 8- to 10-fold higher expression of surface binding. Cells transfected with all three subunits expressed the highest number of cell surface receptors. The binding capacities of cells transfected with $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$ or $\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$ combinations were approximately two-fold higher than the corresponding combinations containing the β_{1-2} subunit. Likewise, cells transfected with $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ or $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$ combinations expressed approximately 2.5-fold more binding sites per cell than the corresponding combinations containing α_{1B-2}. In all cases, co-expression of the α_{2b} subunit with α_{1B} and β_1 increased the surface receptor density compared to cells transfected with only the corresponding α_{1B} and β_1 combinations; approximately 8-fold for $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$, 10-fold for $\alpha_{1B-1}\alpha_{2b}\beta_{1-3}$, 9-fold for $\alpha_{1B-2}\alpha_{2b}\beta_{1-2}$, and 13-fold for $\alpha_{1B-2}\alpha_{2b}\beta_{1-3}$. Thus, comparison of the B_{max} values suggests that the toxin-binding subunit, α_{1B-1} or α_{1B-2}, is more efficiently expressed and

assembled on the cell surface when co-ex-pressed with either the α_{2b} or the β_{1-2} or β_{1-3} subunit, and most efficiently expressed when α_{2b} and β_1 subunits are present.

3. Electrophysiology

Functional expression of $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ and $\alpha_{1B-1}\beta_{1-2}$ subunit combinations was evaluated using the whole-cell recording technique. Transfected cells that had no contacts with surrounding cells and simple morphology were used approximately 48 hours after transfection for recording. The pipette solution was (in mM) 135 CsCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES, and 4 mM Mg-ATP (pH 7.3, adjusted with TEA-OH). The external solution was (in mM) 15 $BaCl_2$, 125 Choline Cl, 1 $MgCl_2$, and 10 HEPES (pH 7.3, adjusted with TEA-OH). ω-CgTx GVIA (Bachem) was prepared in the external solution with 0.1% cytochrome C (Sigma) to serve as a carrier. Control experiments showed that cytochrome C had no effect on the Ba^{2+} current.

The macroscopic electrophysiological properties of Ba^{2+} currents in cells transfected with various amounts of the α_{2b} expression plasmid with the relative amounts of α_{1B-1} and β_{1-2} plasmids held constant were examined. The amplitudes and densities of the Ba^{2+} currents (15 mM $BaCl_2$) recorded from whole cells of these transfectants differed dramatically. The average currents from 7 to 11 cells of three types of transfections (no α_{2b}; 2:1.8:1 [α_{1B-1}:α_{2b}:β_{1-2}] molar ratio; and 2:10.9:1 [α_{1B-1}:α_{2b}:β_{1-2}] molar ratio) were determined. The smallest currents (range: 10 to 205 pA) were recorded when α_{2b} was not included in the transfection, and the largest currents (range: 50 to 8300 pA) were recorded with the 2:10.9:1 ratio of $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ plasmids, the ratio that resulted in near equivalent mRNA levels for each subunit transcript. When the amount of α_{2b} plasmid was adjusted to yield approximately an equal abundance of subunit mRNAs, the average peak Ba^{2+} current increased from 433 pA to 1,824 pA (4.2-fold) with a corresponding increase in average current density from 26 pA/pF to 127 pA/pF (4.9-fold). This increase is in the presence of a 2.7-fold *decrease* in the mass of α_{1B-1} and β_{1-2} expression plasmids in the transfections.

In all transfections, the magnitudes of the Ba^{2+} currents did not follow a normal distribution.

To compare the subunit combinations and determine the effects of α_{2b}, the current-voltage properties of cells transfected with $\alpha_{1B-1}\beta_{1-2}$ or with $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ in either the 2:1.8:1 (α_{1B-1}:α_{2b}:β_{1-2}) molar ratio or the 2:10.9:1 (α_{1B-1}:α_{2b}:β_{1-2}) molar ratio transfectants were examined. The extreme examples of no α_{2b} and 11.25 μg α_{2b} (2:10.9:1 molar ratio) showed no significant differences in the current voltage plot at test potentials between 0 mV and +40 mV ($p<0.05$). The slight differences observed at either side of the peak region of the current voltage plot were likely due to normalization. The very small currents observed in the $\alpha_{1B-1}\beta_{1-2}$ transfected cells have a substantially higher component of residual leak relative to the barium current that is activated by the test pulse. When the current voltage plots are normalized, this leak is a much greater component than in the $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}$ transfected cells and as a result, the current-voltage plot appears broader. This is the most likely explanation of the apparent differences in the current voltage plots, especially given the fact that the current-voltage plot for the $\alpha_{1B-1}\beta_{1-2}$ transfected cells diverge on both sides of the peak. Typically, when the voltage-dependence activation is shifted, the entire current-voltage plot is shifted, which was not observed. To qualitatively compare the kinetics of each, the average responses of test pulses from -90 mV to 10 mV were normalized and plotted. No significant differences in activation or inactivation kinetics of whole-cell Ba^{2+} currents were observed with any combination.

G. Expression of DNA encoding human neuronal calcium channel $\alpha_{1E-3}\alpha_{2B}\beta_{1-3}$ and $\alpha_{1E-1}\alpha_{2B}\beta_{1-3}$ subunits in HEK cells

Functional expression of the $\alpha_{1E-1}\alpha_{2B}\beta_{1-3}$ and $\alpha_{1E-3}\alpha_{2B}\beta_{1-3}$, as well as α_{1E-3} was evaluated using the whole cell recording technique.

1. Methods

Recordings were performed on transiently transfected HEK 293 cells two days following the transfection, from cells that had no contacts with surrounding cells and which had simple morphology.

The internal solution used to fill pipettes for recording the barium current from the transfected recombinant calcium channels was (in mM) 135 CsCl, 10 EGTA, 1 $MgCl_2$, 10 HEPES, and 4 mM Mg-ATP (pH 7.4-7.5, adjusted with TEA-OH). The external solution for recording the barium current was (in mM) 15 $BaCl_2$, 150 Choline Cl, 1 $MgCl_2$, and 10 HEPES and 5 TEA-OH (pH 7.3, adjusted with TEA-OH). In experiments in which Ca^{2+} was replaced for Ba^{2+}, a Laminar flow chamber was used in order to completely exchange the extracellular solution and prevent any mixing of Ba^{2+} and Ca^{2+}. ω-CgTx GVIA was prepared in the external solution with 0.1% cytochrome C to serve as a carrier, the toxin was applied by pressurized puffer pipette. Series resistance was compensated 70-85% and currents were analyzed only if the voltage error from series resistance was less than 5 mV. Leak resistance and capacitance was corrected by subtracting the scaled current observed with the P/-4 protocol as implemented by pClamp (Axon Instruments).

2. Electrophysiology Results

Cells transfected with $\alpha_{1E\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$ or $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ showed strong barium currents with whole cell patch clamp recordings. Cells expressing $\alpha_{1E\text{-}3}\alpha_{2B}\beta_{1\text{-}3}$ had larger peak currents than those expressing $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$. In addition, the kinetics of activation and inactivation are clearly substantially faster in the cells expressing α_{1E} calcium channels. HEK 293 cells expressing $\alpha_{1E\text{-}3}$ alone have a significant degree of functional calcium channels, with properties similar to those expressing $\alpha_{1E}\alpha_{2b}\beta_{1\text{-}3}$ but with substantially smaller peak barium currents. Thus, with α_{1E}, the α_2 and β_1 subunits are not required for functional expression of α_{1E} mediated calcium channels, but do substantially increase the number of functional calcium channels.

Examination of the current voltage properties of $\alpha_{1E}\alpha_{2b}\beta_{1\text{-}3}$ expressing cells indicates that $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ is a high-voltage

activated calcium channel and the peak current is reached at a potential only slightly less positive than other neuronal calcium channels also expressing α_{2b} and β_1, and α_{1B} and α_{1D}. Current voltage properties of $\alpha_{1E\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$ and $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ are statistically different from those of $\alpha_{1B\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$. Current voltage curves for $\alpha_{1E\text{-}1}\alpha_{2b}\beta_{1\text{-}3}$ and $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ peak at approximately +5mV, as does the current voltage curve for $\alpha_{1E\text{-}3}$ alone.

The kinetics and voltage dependence of inactivation using both prepulse (200 ms) and steady-state inactivation was examined. α_{1E} mediated calcium channels are rapidly inactivated relative to previously cloned calcium channels and other high voltage-activated calcium channels. $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ mediated calcium channels are inactivated rapidly and are thus sensitive to relatively brief (200 ms) prepulses as well as long prepulses (>20s steady state inactivation), but recover rapidly from steady state inactivation. The kinetics of the rapid inactivation has two components, one with a time constant of approximately 25 ms and the other approximately 400 ms.

To determine whether α_{1E} mediated calcium channels have properties of low voltage activated calcium channels, the details of tail currents activated by a test pulse ranging -60 to +90 mV were measured at -60 mV. Tail currents recorded at -60 mV could be well fit by a single exponential of 150 to 300 μs; at least an order of magnitude faster than those typically observed with low voltage-activated calcium channels.

HEK 293 cells expressing $\alpha_{1E\text{-}3}\alpha_{2b}\beta_{1\text{-}3}$ flux more current with Ba^{2+} as the charge carrier and currents carried by Ba^{2+} and Ca^{2+} have different current-voltage properties. Furthermore, the time course of inactivation is slower and the amount of prepulse inactivation less with Ca^{2+} as the charge carrier.

While the invention has been described with some specificity, modifications apparent to those with ordinary skill in the art may be made without departing from the scope of the invention. Since such modifications will be apparent to

those of skill in the art, it is intended that this invention be limited only by the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA fragment, comprising a sequence of nucleotides that encodes an α_1 subunit selected from the group consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1C-2} and α_{1E-3}.

2. The DNA fragment of claim 1, wherein the α_1 subunit is α_{1A-1} or α_{1A-2}.

3. The DNA fragment of claim 1, wherein the α_1 subunit is α_{1E-1} or α_{1E-3}.

4. The DNA fragment of claim 1, wherein the α_1 subunit is α_{1C-2}.

5. An isolated DNA fragment, comprising a sequence of nucleotides that encodes a β subunit selected from the group consisting of β_2, β_3 and β_4.

6. The DNA fragment of claim 5, wherein the subunit is a β_{2C}, β_{2D} or β_{2E} subunit.

7. The DNA fragment of claim 5, wherein the subunit is a β_3 subunit.

8. The DNA fragment of claim 7, wherein the subunit is a β_{3-1} subunit.

9. The DNA fragment of claim 5, wherein the subunit is a β_4 subunit.

10. The DNA fragment of claim 9, wherein the subunit has an amino acid sequence set forth in SEQ ID No. 28.

11. A eukaryotic cell, comprising heterologous DNA that encodes an α_1 subunit selected from the group of subunits consisting of α_{1A-1}, α_{1A-2}, α_{1C-2}, α_{1E-1}, and α_{1E-3}.

12. A eukaryotic cell, comprising heterologous DNA that encodes an α_1 subunit and heterologous DNA that encodes a β subunit, wherein at least one subunit is selected from the group of subunits consisting of α_{1A-1}, α_{1A-2}, α_{1C-2}, α_{1E-1}, α_{1E-3}, β_{2C}, β_{2D}, β_{2E}, β_{3-1}, a β_4 subunit.

13. The eukaryotic cell of claim 12, wherein the β subunit is a β_2 subunit.

14. The eukaryotic cell of claim 12, wherein the β subunit is a β_4 subunit.

15. The eukaryotic cell of claim 11, selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, and mouse L cells.

16. The eukaryotic cell of claim 12 selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, and mouse L cells.

17. A eukaryotic cell with a functional, heterologous calcium channel, produced by a process comprising:

introducing into the cell heterologous nucleic acid that encodes an α_1-subunit of a human calcium channel, wherein:

the α_1 subunit is selected from the group consisting of α_{1A-1}, α_{1A-2}, α_{1C-2}, α_{1E-1} and α_{1E-3};

the heterologous calcium channel contains at least one subunit encoded by the heterologous nucleic acid; and

the only heterologous ion channels are calcium channels.

18. A eukaryotic cell with a functional, heterologous calcium channel, produced by a process comprising:

introducing into the cell nucleic acid that encodes an α_1 subunit of a human calcium channel and introducing into the cell nucleic acid that encodes a β subunit of a human calcium channel, wherein:

at least one of the subunits is s elected from the group consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1E-3}, β_{2C}, β_{2D}, β_{2E}, a β_3 and a β_4 subunit;

the heterologous calcium channel contains at least one subunit encoded by the heterologous nucleic acid; and

the only heterologous ion channels are calcium channels.

19. The eukaryotic cell of claim 17 selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, mouse L cells and amphibian oöcytes.

20. The eukaryotic cell of claim 18 selected from the group consisting of HEK 293 cells, Chinese hamster ovary cells, African green monkey cells, mouse L cells and amphibian oöcytes.

21. The eukaryotic cell of claim 18, wherein the β subunit is a β_2, β_3 or β_4 subunit of a human calcium channel.

22. The eukaryotic cell of claim 18, wherein the calcium channel includes an α_{2b} subunit of a human calcium channel, an α_{1B-1} subunit of a human calcium channel and a β_3 subunit of a human calcium channel.

23. The eukaryotic cell of claim 18, wherein the calcium channel includes an α_{1B-1}, α_{2b}, and a β_{1-2} subunit, or an α_{1B-1}, α_{2b}, and a β_{1-3} subunit, or an α_{1B-2}, α_{2b}, and a β_{1-3} subunit, or an α_{1A-2}, α_{2b}, and a β_{3-1} subunit, or a α_{1B-1}, α_{2b}, and an β_{3-1} subunit.

24. The eukaryotic cell of claim 18, wherein the calcium channel contains an α_{2b} subunit of a human calcium channel, an α_{1B} or an α_{1D} subunit of a human calcium channel and a β_{1-1}, β_{1-2} or β_{1-3} subunit of a human calcium channel.

25. A method for identifying a compound that modulates the activity of a calcium channel, comprising;

suspending a eukaryotic cell that has a functional, heterologous calcium channel, in a solution containing the compound and a calcium channel-selective ion:

depolarizing the cell membrane of the cell; and

detecting the current flowing into the cell,

wherein:

the heterologous calcium channel includes at least one human calcium channel subunit encoded by DNA or RNA that is heterologous to the cell;

at least one subunit is selected from the group consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1E-3}, α_{1C-2}, β_{2C}, β_{2D}, β_{2E}, a β_3 subunit and a β_4 subunit;

the current that is detected is different from that produced by depolarizing the same or a substantially identical cell in the presence of the same calcium channel selective ion but in the absence of the compound.

26. The method of claim 25, wherein the heterologous DNA or RNA encodes a β_3 subunit.

27. The method of claim 26, wherein the heterologous DNA or RNA encodes a β_4 subunit.

28. A subunit-specific antibody selected from the group consisting of antibodies that bind to an α subunit type or α subunit subtype of a human calcium channels, wherein the subunit is an α_1 subunit.

29. The antibody of claim 28, wherein antibody is subtype specific and the α_1 subunit is α_{1A}, α_{1E} and α_{1B}.

30. An RNA or single-stranded DNA probe of at least 16 bases in length comprising at least 16 substantially contiguous bases from nucleic acids that encode a subunit of a human calcium channel selected from the group of subunits consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1C-2}, α_{1E-3}, β_{3-1}, β_{2C}, β_{2D}, β_{2E} and β_4.

31. The probe of claim 30 that contains at least 30 bases that are from nucleic acids that encode a subunit of a human calcium channel selected from the group of subunits consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1C-2}, α_{1E-3}, β_{3-1}, β_{2C}, β_{2D}, β_{2E} and β_4 subunits.

32. A method for identifying nucleic acids that encode a human calcium channel subunit, comprising hybridizing under conditions of at least low stringency a probe of claim 30 to a library of nucleic acid fragments, and selecting hybridizing fragments.

33. A method for identifying cells or tissues that express a calcium channel subunit-encoding nucleic acid, comprising hybridizing under conditions of at least low stringency a probe of claim 30 with mRNA expressed in the cells or tissues or cDNA produced from the mRNA, and thereby identifying cells or tissue that express mRNA that encodes the subunit.

34. A substantially pure human calcium channel subunit selected from the group consisting of α_{1A-1}, α_{1A-2}, α_{1E-1}, α_{1C-2}, α_{1E-3}, β_{3-1}, β_{2C}, β_{2D}, β_{2E} and β_4.